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Ana
Parga Martínez

PhD Thesis

Quorum sensing and oral biofilm: an alternative for the prevention and treatment of oral diseases

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DOCTORAL THESIS

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Ana Parga Martínez

INTERNATIONAL PHD SCHOOL OF THE UNIVERSITY OF SANTIAGO DE COMPOSTELA

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Quorum sensing and oral biofilm: an alternative for the prevention and treatment of oral diseases

Dª. Ana María Otero Casal

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Abbreviations

AAA model: Amsterdam Active Attachment model.

AE: aerobiosis.

AHLs: *N*-acyl-homoserine lactones.

AIs: autoinducers.

AI-2: autoinducer-2.

AIPs: autoinducer peptides.

AN: acetonitrile.

ANE: anaerobiosis.

BHI : Brain-Heart Infusion.

cDNA: complementary DNA.

CLSM: confocal-laser scanning microscopy.

CSP: competence-stimulating peptide.

CV: crystal violet.

DM: dichloromethane.

EA: ethyl acetate.

gDNA: genomic DNA.

HA: hydroxyapatite.

HPLC-MS: high-performance liquid chromatography–mass spectrometry.

HSL: homoserine lactone.

HTS: high throughput sequencing.

IQR: interquartile range.

LB: Luria-Bertani.

LDA: Linear Discriminant Analysis.

MS: mass spectrometry.

PBS: phosphate buffered saline.

PCR: polymerase chain reaction.

qPCR: quantitative polymerase chain reaction.

QS: Quorum Sensing.

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QSI: Quorum Sensing Inhibition.

QQ: Quorum Quenching.

RT: room temperature.

RT-qPCR: real-time quantitative polymerase chain reaction.

SCH-K1: Schaedler culture medium, supplemented with vitamin K1 (0.1 mg/L).

SD: standard deviation.

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

SEM: standard error of the mean.

Sub-MIC: sub-minimum inhibitory concentration.

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Introduction

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The contents of the introduction are partially presented in a book chapter titled “Ecological modulation of oral biofilms: novel approaches to treat and prevent oral diseases”, and authored by Ana Parga[1], and Ana Otero[1].

The manuscript “Ecological modulation of oral biofilms: novel approaches to treat and prevent oral diseases” has been accepted and soon to be published as a chapter in the book “Oral Biofilms in Health and Disease”, part of the book series “Springer Series on Biofilms” (ISSN: 1863-9607) edited by *Springer*.

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[1] Department of Microbiology and Parasitology, CIBUS-Faculty of Biology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain

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1. Relevance of human oral health

In recent years, oral health has been increasingly described as an important factor contributing to general health and quality of life. According to the World Health Organization (WHO), in the year 2019, 2000 million people worldwide had untreated caries of permanent teeth, and 1000 million people suffered severe periodontal disease (World Health Organization, 2022). Dental caries is the most common non-communicable disease, with more than one third of the population being affected by untreated caries. It is also the single most common childhood disease, with untreated caries of deciduous teeth affecting 500 million children in the year 2019 (World Health Organization, 2022). Additionally, the WHO estimates that severe periodontal disease is the main cause of loss of dental pieces in adults. The prevalence of periodontal disease has increased by 24% between 1990 and 2019, despite the efforts invested in its treatment and prevention. Prevalence of severe periodontal disease increases with age. In the current global context, the progressively larger ageing populations, and the increase in longevity, point towards a future with an even higher global burden of severe periodontal disease (World Health Organization, 2022). Furthermore, potential connections between oral diseases and other, more severe, chronic diseases, have been increasingly pointed out (Seitz et al., 2019). Strong evidence exists for a link between severe periodontal disease and systemic inflammatory disorders (Hajishengallis, 2015; Hajishengallis and Chavakis, 2021), diabetes mellitus (D'Aiuto et al., 2017), and cardiovascular disease (Pasqualini et al., 2012; Batty et al., 2018). Furthermore, periodontitis-associated microbiota has been proposed as a risk factor for Alzheimer's disease (Pritchard et al., 2017). Therefore, more research on the mouth ecosystem and the interactions with and within the oral microbiome is crucial, not only to increase our knowledge of oral disease development, but also to understand the impact of oral health on general health and its relationship with other diseases.

Despite the prevalence, cost and severity of oral diseases, their control largely relies on the use of antimicrobials. The increase in resistance towards these compounds makes imperative the search for new, effective strategies for the prevention and treatment of oral diseases. This search must be made in the context of microbial oral ecology, approaching the problem from the perspective of the existent bacterial interactions. The development of strategies aiming to modulate the processes of intercellular communication is proposed as a promising lead to battle this growing health problem.

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2. Oral biofilm and its relation to health and disease

The mouth is one of the most biologically complex sites of the human body. One of the main elements accounting for this complexity is its direct connection with the external environment. The continuous interaction of the mouth with the outer world, makes the oral cavity an extremely microbiologically rich environment, and also the first entry site for microbial pathogens (Marsh et al., 2016). More than 700 bacterial species have been identified inhabiting different niches within the oral cavity (Aas et al., 2005). Anatomically, the mouth contains surfaces covered by a shedding epithelium (tongue, cheeks, hard and soft palate, gingiva, or tonsils), and non-shedding surfaces (dental enamel), which have associated different microbiota profiles (Simón-Soro et al., 2013). Non-shedding surfaces allow the building of more permanent bacterial structures; the oral biofilm (Quirynen and Bollen, 2005). Oral biofilms comprise bacteria embedded in a matrix of extracellular DNA and other polymers of host and bacterial origin (Marsh, 2006). Internally, biofilms display spatial and functional organisation, and bacteria within them show distinct phenotypes than those of bacteria growing in planktonic states (Donlan and Costerton, 2002; Marsh et al., 2016). Some of these phenotypes are of utmost clinical relevance as, for example, biofilms are estimated to be 1000-fold more tolerant to antimicrobials as compared with planktonic cells (Gilbert et al., 1997).

Oral biofilm formation is a dynamic and continuous process, involving constant reorganisation of its constituents (Kolenbrander et al., 2006). Bacteria, rather than adhering directly to teeth, interact with the acquired pellicle that is formed onto any surface exposed to the oral cavity. This acquired pellicle is mostly composed of salivary proteins, glycoproteins, and lipids, and conditions the bacteria that will adhere to it (Marsh et al., 2016). Bacteria can come in contact with surfaces covered by the acquired pellicle either in a passive way, transported by the salivary flow, or actively, if they are motile. This first contact is driven by weak physicochemical forces, which rapidly evolve as bacteria employ surface molecules such as adhesins to engage with receptors in the acquired pellicle (Jenkinson and Lamont, 1997). The first microorganisms that appear on the tooth surface are termed early colonisers and are mainly coccus, specifically, streptococci, followed shortly by *Actinomyces, Haemophilus,* and *Neisseria.* Strict anaerobes are observed in low proportion at this stage (Marsh et al., 2016). Biofilm evolves in a process known as microbial succession, in which oral biofilm composition becomes more diverse as other taxa are incorporated. This process is facilitated by the metabolic activity of early colonisers, which make metabolites available to other bacteria to use as primary

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nutrient sources (Marsh et al., 2016). To persist within the biofilm, new taxa must adhere to either the acquired pellicle, or to the surface of early coloniser bacteria, a process termed coaggregation (Kolenbrander, 1988). Fusobacteriota (former Fusobacteria) have been described as pivotal in the coaggregation process, serving as a "bridge" between early colonisers with which they aggregate, and late colonisers that adhere to Fusobacteria. Thus, Fusobacteria contribute to oral biofilm formation and greatly condition the composition of such structures (Bradshaw et al., 1998; Thurnheer et al., 2019). As biofilm maturation continues, microbial diversity increases even more, and the extracellular matrix of the biofilm begins to develop. The matrix is mainly composed by soluble and insoluble glucans, fructans, proteins and extracellular DNA (Allison, 2003). The biofilm matrix role is not restricted to that of a structural support, but also protects the biofilm from environmental stresses, from desiccation to antimicrobial compounds, by retaining water into its structure, and chemically and physically restricting the penetration of several elements, such as charged antimicrobials (Jakubovics et al., 2021). Inside the biofilm, highly diverse microhabitats are formed, facilitating the contact between different bacteria. Such contact will result in antagonistic and synergistic interactions, as well as horizontal gene transfer, and cell-cell signalling processes such as Quorum Sensing, which enable bacteria to coordinate their activities, and that will be addressed later in this introduction. These processes drive the evolution of cells within biofilms, and account for the emergence of more virulent phenotypes. Once biofilms are mature, some bacteria will free themselves to colonise other niches in a process termed biofilm dispersal, starting a new biofilm formation cycle (Marsh et al., 2016).

Oral health is currently understood as the maintenance of a diverse microbiome that has established beneficial relationships both within itself and with the host (Zarco et al., 2012). A healthy oral microbiome will act as a barrier against opportunistic pathogens and help maintaining homeostasis in the oral cavity through metabolic and immunomodulatory activities (He et al., 2011; Devine et al., 2015). The equilibrium necessary for such a healthy microbiome is maintained through competitive and cooperative microbial interactions (Marsh, 1994, 2003). These interactions take place between the commensal beneficial microbiota and sometimes species associated with disease. Keystone pathogens are disease-associated species found in low proportion in the oral cavity, but that can have great impact on the local microhabitat, by setting the conditions that alter the equilibrium of the biofilm (Hajishengallis et al., 2012). Once the biofilm microhabitat has been altered, some of the commensal bacteria will

disproportionally thrive in it, further supporting the progression of the disease. These commensals are termed pathobionts (Costalonga and Herzberg, 2014; Jiao et al., 2014). It is relevant to highlight that the presence of both keystone pathogens and pathobionts is not detrimental *per se* to oral health, but rather the combination of their activities and their surrounding environment, as well as host genetics and habits that account for the detrimental effects on oral health (Belda-Ferre et al., 2012; Duran-Pinedo and Frias-Lopez, 2015; Yost et al., 2015). Together, these elements can result in a loss of microbiome equilibrium, i.e., dysbiosis, leading to a pathogenic biofilm (Marsh, 1994, 2003; Hajishengallis et al., 2012). The most common pathologies resulting from such dysbiosis are caries and periodontitis. Therefore, caries and periodontitis, the two major oral diseases, result from a regime shift driven by carbohydrate consumption and acidification and plaque accumulation, respectively (Rosier et al., 2018).

2.1. Dental caries

Dental caries is a polymicrobial disease which starts with a disturbance in a healthy oral microbiota caused by an increase in fermentable sugars and carbohydrates intake (Simón-Soro and Mira, 2015). Metabolism of these compounds by bacteria such as *Streptococcus mutans* releases organic acids into the oral environment. Continued acid release lowers the pH of the surrounding biofilm, further selecting for acidophile bacteria that can thrive in these conditions. Through these activities, the healthy bacterial community within the oral biofilm eventually shifts towards a cariogenic population (Bradshaw and Lynch, 2013; Sheiham and James, 2015; Boisen et al., 2021). The low pH and the cariogenic bacterial metabolism, leads to the demineralization of dental hard tissues and eventually to the exposure of the dental pulp. Bacterial infection of the pulp rapidly evolves into inflammation of the surrounding tissues of the tooth (apical periodontitis), and if untreated, can ultimately cause tooth loss.

Acidogenic bacteria such as *S. mutans*, able to induce important changes in the biofilm it inhabits, are found in low proportion in the oral cavity (Simón-Soro et al., 2014). As a result, this species has been classically defined as a keystone pathogen in dental caries. Nevertheless, in recent years, more importance has been given to the functional activities occurring within oral biofilms, and it is now acknowledged that the phenotypic characteristics of a biofilm at a given moment can have a greater influence in the progression of dental caries than the microbiome structure (Takahashi and Nyvad, 2011; Duran-Pinedo and Frias-Lopez, 2015;

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Simón-Soro and Mira, 2015). Host susceptibility completes this picture of dental caries, and is defined by the unique combination of genetics and environment of each person, and that determines the unique progression of the disease in an individual (Selwitz et al., 2007; Lif Holgerson et al., 2015).

2.2. Periodontal disease

Periodontal diseases are also of polymicrobial nature, and can be classified into gingivitis, a reversible state of the disease, and periodontitis, an irreversible state characterised by chronic inflammation and bone resorption. Periodontal disease begins with an increase in subgingival biofilm formation and in the number of pathobiont species found in the periodontal pocket (Hajishengallis et al., 2011). These species are commonly proteolytic and their activity modifies the biofilm microhabitat, causing an inflammation of the gingival tissues that initiates the diseased state. The inflammation drives the activation of the immune responses of the host, which provides substrates that are metabolised by pathogenic bacteria, helping the progression of the disease (Hajishengallis, 2014).

Two groups of bacteria have been classically linked to the initiation and progression of periodontal disease; the "red complex" bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*), found most frequently in deeper periodontal pockets, and the "orange complex" (*Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, and *Parvimonas micra*), also found in deep periodontal pockets, and usually preceding the establishment of "red complex" bacteria (Socransky et al., 1998; Marsh et al., 2016). These species have been found both in healthy and disease sites, highlighting the importance of the environmental conditions to shift the equilibrium of the oral biofilm to a diseased state. As for dental caries, it is now considered that the combination of functional activities within biofilms is as important as their bacterial composition, when trying to understand biofilm responses and disease progression (Duran-Pinedo and Frias-Lopez, 2015; Yost et al., 2015).

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3. Modulating oral biofilm formation and development

Traditional oral treatments are based on correcting dietary habits, improving oral hygiene, and administration of fluorinated compounds for caries control, and mechanical removal of infected tissues, in some cases in combination with antibacterial agents, for periodontal diseases. These treatments, aiming at resetting the dental plaque, are often ineffective, partially due to the stability of the oral microbiota, which restores rapidly after treatment (Zaura et al., 2015), and to the difficulties in reaching and penetrating dysbiotic biofilms. Although the use of antiseptics such as chlorhexidine gluconate or triclosan has some unarguable clinical benefits (revised by Serrano et al. (2015)), these compounds may have undesirable side effects, including the down-regulation of oral keratinocytes and fibroblasts (Fernandes et al., 2018) or the reduction of the protective barrier against pathogens provided by the commensal microbiota (He et al., 2011). For chlorhexidine, the most common oral antiseptic, it has been demonstrated that its indiscriminate antimicrobial action may cause major shifts in oral microbiota and metabolic activity, increasing the abundance of pathobionts, leading to more acidic conditions, or lowering nitrate availability (Bescos et al., 2020; Chatzigiannidou et al., 2020).

In this perspective, there is an increasing need for the development of novel, more targeted treatments, and preventive measures that should aim at maintaining a healthy, microbially diverse resident microbiome, promoting its resilience to the disease drivers and curving the self-activation loops that drive to dysbiosis (Marsh et al., 2015; Fernandes et al., 2018; Philip et al., 2018; Rosier et al., 2018; Wade, 2021). Given the prevalent functional redundancy of microbial groups in the oral environment, the novel strategies should also target key metabolic activities and the expression of virulence factors rather than focus on specific groups of microbes. In this section of the introduction, we will provide a general view of the novel strategies being explored for the targeted modulation of the oral microbiota. Some of these strategies seem to be already playing an important role in the oral environment, such as antimicrobial peptides (AMPs), bacteriophages, and Quorum Sensing Inhibitors. Pre and probiotics also represent a more ecological approach targeting the re-establishment of the holobiont equilibrium.

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3.1. Prebiotics

Prebiotics are non-digestible substrates that are selectively utilized by commensal microorganisms conferring a health benefit to the host (Gibson et al., 2017). The use of prebiotics for improving oral health has been focused mainly on caries prevention and treatment (Zaura et al., 2017; Montero et al., 2019). A caries-prone oral environment provides a low pH and high availability of lipidic metabolites and dietary carbohydrates. These characteristics favour the proliferation of bacteria that will ferment those carbohydrates yielding acidic products, like lactic acid, that result in the demineralization of the enamel (Marsh et al., 2016; Philip et al., 2018; Nascimento, 2019). Additionally, an elevated carbohydrate intake can lead to a positive feedback acidification loop driving the selection of acidophilic, cariogenic pathogens (Rosier et al., 2018).

In the last decades, cumulative evidence points to modulating the alkalinogenic potential of dental biofilms as a promising strategy for caries control. In a healthy biofilm, the metabolism of amino acids, peptides, proteins, and urea leads to the net production of alkali. Alkali production helps to increase the pH after acid production resulting from the dietary intake of fermentable carbohydrates. Thus, as we have introduced above, when this equilibrium is tipped towards a predominance of acid production, caries can occur. One of the prebiotic approaches to modulate this shift is the administration of compounds that, when metabolized, raise the pH of their surrounding environment, counteracting the acidogenic effect of cariogenic bacteria.

Arginine is an amino acid found in saliva in free form and in salivary peptides and proteins (VanWuyckhuyse et al., 1995; Nascimento, 2019). It has been identified as the main agent responsible for the increase in salivary pH, and it has been proposed as an agent to promote oral health (Nascimento, 2019). Arginine is produced *de novo* from citrulline and from protein turnover, but it also enters the mouth with the dietary intake. The microbial degradation of arginine comprises several routes, the most common one being the arginine deiminase system (ADS) that yields citrulline, ornithine, CO_2, ATP, and ammonia (Nascimento, 2019) (Figure 1). This route is present in certain bacteria in supragingival biofilms, such as streptococci of the mitis group (*Streptococcus sanguinis, Streptococcus gordonii, Streptococcus parasanguinis, and Streptococcus mitis*), and some lactobacilli and *Actinomyces* strains (Liu et al., 2012; Marsh et al., 2016). This production of ammonia results in a pH increase in the surroundings of these oral bacteria and protects them against acid. Additionally, the neutralization of the biofilm

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favours the persistence of ADS positive bacteria and makes them competitive against aciduric and cariogenic microorganisms (Burne and Marquis, 2000). In fact, low levels of arginine deiminase activity in saliva and plaque have been proposed as indicators for caries risk in adult subjects (Bijle et al., 2018). Arginine has been administered with fluoride in dentifrices for over a decade with promising results and without negative side effects (Montero et al., 2019). Arginine seems to have synergistic effects when combined with fluoride, as this combination can suppress exopolysaccharide production, inhibiting biofilm formation (Zheng et al., 2015), and it is generally an effective approach to restoring the oral microbial equilibrium (revised in Zaura and Twetman (2019)). The arginine-fluoride combination has shown to inhibit *Streptococcus mutans* while favouring *S. sanguinis,* a species that is ADS positive and thus can use the available arginine to produce alkali (Zheng et al., 2015). At the same time, maintaining those oral niches colonised by streptococci exerts an ecological pressure against colonisation by bacteria that thrive in alkalinogenic environments, such as the periodontopathogen *Porphyromonas gingivalis* (Zheng et al., 2015). However, the long-term impact of this strategy has yet to be confirmed. A feature to consider regarding the use of arginine as a prebiotic is that this amino acid is also a substrate of the nitric oxide synthase, i.e., a precursor of nitric oxide (NO) (Figure 1). NO can act as an antimicrobial agent but also be involved in the pathogenesis of periodontitis (Parwani and Parwani, 2015). The delicate equilibrium between NO beneficial and detrimental effects on oral health should be further studied in the context of ecological modulations in this environment, as addressed later in this section regarding the use of nitrate as a prebiotic. With this in mind, prebiotic administration in caries patients at risk of developing other oral diseases or with other oral diseases already ongoing must be done cautiously, choosing the most beneficial strategy for each patient, and avoiding the risk of promoting further dysbiosis states.

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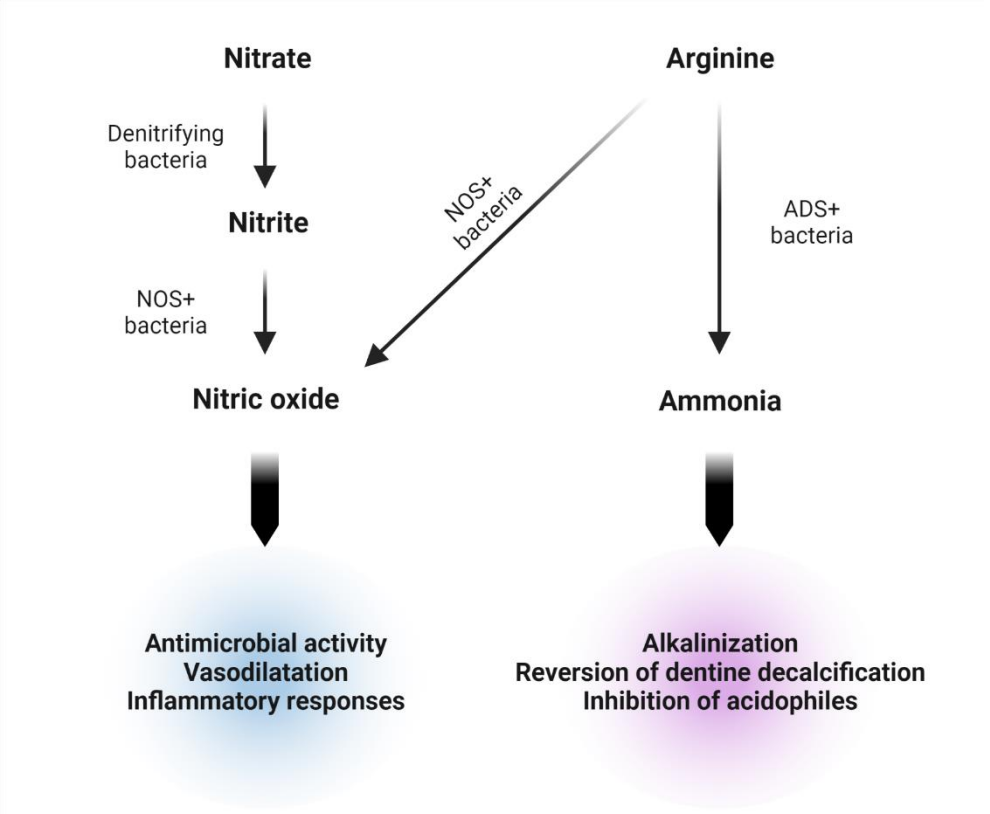


Figure 1. Convergence of different bacterial metabolic pathways in the production of nitric oxide. Nitrate reduction can lead to nitric oxide generation (left). Arginine plays a role in pH homeostasis via the arginine deiminase (ADS) pathway (right), and in nitric oxide synthesis, via the nitric oxide synthase (NOS) pathway. Modified from Parga and Otero (in press)**.**

Although its applicability as a prebiotic has been only recently suggested, evidence of the role of urea metabolism in acid neutralization in dental plaque goes way back, as urea-containing mouthwashes began to be used decades ago to reduce lactic acid production in the mouth and inhibit the development of new caries in individuals with active caries (Stephan, 1943; Kesel, 1948). Similar to the metabolism of arginine, the metabolism of urea mediated by the enzyme urease results in the production of ammonia and the subsequent increase in the pH of the biofilms (Marsh et al., 2016). This metabolic route is present in some oral bacteria such as streptococci from the salivarius group (*Streptococcus salivarius* and *Streptococcus vestibularis*), *Actinomyces naeslundii*, and *Haemophilus* spp. (Liu et al., 2012; Marsh et al., 2016). Expression of urease is up-regulated at low pH as a response to pH stress in these bacteria (Marsh et al., 2016). In addition, several studies have linked low levels of urease activity in supragingival plaque with caries (Shu et al., 2007; Nascimento et al., 2009; Morou-Bermudez et al., 2011). Despite this evidence, the supplementation with urea as a prebiotic for anticaries

purposes has not been widely studied since and needs further assessments to establish its efficacy and safety (Zaura and Twetman, 2019).

Nitrate is obtained via dietary intake, mainly from vegetables, and influences the oral microbiota of the host (Marsh et al., 2016). Nitrate is absorbed into the bloodstream, but an important portion is concentrated back in the saliva, where it will be reduced to nitrite by facultative anaerobic oral bacteria, with members of the genera *Neisseria, Veillonella, Actinomyces,* and *Rothia* showing the greatest nitrate reductase activities (Marsh et al., 2016). Nitrate reduction depends entirely on the commensal oral bacteria, as humans cannot perform this reaction (Marsh et al., 2016). Therefore, due to intraindividual microbiome variation, not all hosts will have the same net nitrate-reducing capacity (Rosier et al., 2020b). In fact, nitrate-reducing bacteria are more common in individuals with less caries episodes (Doel et al., 2004). Prebiotic supplementation with nitrate can help increase nitrate-reducing bacteria over time, thus increasing available nitrite and NO (Rosier et al., 2020b, 2020a) (Figure 1). Among its systemic beneficial effects, nitrite helps lowering blood pressure (Marsh et al., 2016), and NO exerts antimicrobial activities against exogenous bacteria (Marsh et al., 2016). Nevertheless, as stated above, NO can also promote periodontitis progression (Parwani and Parwani, 2015). Although more investigations are needed, studies show that nitrate supplementation prevents the pH drops resulting from carbohydrate metabolism (Rosier et al., 2020b), pointing to the potential efficacy of this prebiotic strategy. Prebiotic nitrate has been proven to change oral microbiota by reducing the abundance of genera related to caries, halitosis, and periodontal disease and inducing a more efficient nitrate reduction in the oral cavity (Rosier et al., 2020b). The reduction of halitosis-related genera could be explained by the differential energetical benefits of nitrate versus sulphate reduction. The latter is less efficient, and thus nitrate availability should limit sulphate metabolism, which yields volatile sulphur products related to halitosis (Rosier et al., 2020a). It should be noted that nitrate and nitrite concentrations in saliva have been reported to be significantly higher in periodontitis patients (Sánchez et al., 2014), which can be the result of a host defence response, as it is known that salivary glands can respond to inflammation processes, such as periodontitis, by increasing salivary protective characteristics (Henskens et al., 1996). One interesting aspect to consider when administering nitrate as a prebiotic would be its delivery through increased dietary nitrate-rich vegetables. The alkalinity of nitrate-rich foods and the presence of co-factors required for the reduction of nitrate

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could enhance the activity of nitrate-reducing bacteria, thus increasing the viability of this strategy.

Other compounds studied because of their cariostatic potential are sugar substitutes such as sugar alcohols. Sugar alcohols, such as xylitol, are a type of low molecular weight polyols that cannot be metabolized by most bacteria in dental plaque (Matsukubo and Takazoe, 2006; Marsh et al., 2016). They are very popular as sugar substitutes in food products and beverages, as they confer moderated sweetness but do not translate into sugar spikes after consumption (Matsukubo and Takazoe, 2006). The latter characteristic, added to the reduction in available fermentable carbohydrates, has set the basis for considering sugar alcohols as prebiotics for caries control. The action mechanisms behind the cariostatic effects of sugar alcohols include the increase in buffering capacity and pH of the oral biofilm and the restoration of the enamel de-/remineralization equilibrium (Matsukubo and Takazoe, 2006). Specific action mechanisms on cariogenic bacteria, specifically mutans streptococci, including inhibiting insoluble glucan synthesis from sucrose and decreasing mutans streptococci cell counts in the mouth (Philip et al., 2018). Nevertheless, even for the most studied sugar alcohols used as sugar substitutes, i.e., xylitol and erythritol, effects on caries control remain overall inconclusive (Philip et al., 2018; Söderling and Pienihäkkinen, 2020). Moreover, it has been brought to attention that the cariostatic results obtained with chewing gums containing sugar alcohols cannot be wholly attributed to the effect of said compounds but at least partially to the salivary flow stimulation derived from the chewing itself, even when the gum does not contain sugar alcohols (Edgar, 1998). Moreover, the low compliance in patients limits the feasibility of sugar alcohols as prebiotic agents because of the long-term daily use required (Gold, 2016).

The strategy that has been proven more effective in the search for prebiotic agents to maintain oral health is the combined administration of prebiotics and probiotics so that the promotion of health-associated or beneficial bacteria is even higher (Kojima et al., 2016). Nevertheless, the application of personalised treatments for either solo or combined approaches has been recommended (Zaura et al., 2017). The complexity of the oral cavity is multiplied when studying different individuals, as each has its particular ecosystem. Moreover, regarding the previously commented approaches, the acid/alkali equilibrium in the oral cavity is highly dependent on the patient, as responses to changes in pH shifts have also been described to be variable between individuals (Zaura et al., 2017). Supplementation with exogenous molecules in the oral context will produce unique responses in each case. As the ulterior motive for

prebiotic therapy is to ameliorate the health status of the host, choices on the specific treatment to recommend must be made on a personalised basis, together with pre-treatment evaluations of risk factors for developing oral diseases different from the ones being targeted with the administration of the prebiotic.

3.2. Probiotics

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Their application for oral health has been gaining attention for the past decades, and several strategies have been tested (Zhang et al., 2022). One of the principles underlying probiotic use is that the probiotic microorganisms should belong to the natural microbiota to establish themselves as residents in the targeted environment (Meurman, 2005). Thus, they would occupy the desired ecological niche so that other less beneficial species cannot colonise it or even displace these potentially harmful microorganisms when infection or disease is already ongoing (Meurman, 2005). The aim of a probiotic is to help achieve and maintain the ecological equilibrium. Although the complete picture of how probiotics help restore the ecological balance is not fully known, several action mechanisms have been described (Meurman, 2005; Stamatova and Meurman, 2009) (Table 1).

The first direct effect of probiotic administration is the competition with other bacteria. This competition takes place both in terms of physical emplacement in a specific ecological niche and as a competition for the available resources found there (Montero et al., 2019; Wade, 2021). Once the probiotic is adhered to an oral surface or integrated into the oral biofilm, it will modulate its surrounding environment by modifying the adhesion of other bacteria to oral surfaces and in between them, i.e., modulating biofilm formation (Montero et al., 2019). This modulation can be achieved through the intrinsic characteristics of the probiotic; this is the case when the cells possess surface components that prevent harmful bacteria from adhering to them. Another strategy for hampering pathogenic biofilm formation is the selection of probiotic bacteria that produce adhesion inhibitors (Meurman, 2005; Stamatova and Meurman, 2009). While several examples of probiotics inhibiting periodontopathogen growth or reducing cell counts have been described, most of the mechanisms behind these effects are still unknown. A commonly hypothesised action mechanism of probiotics is the production of antimicrobial molecules (Table 1). There are some reports on the production of enzymes with anti-biofilm

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activity (i.e., lipase production (Jaffar et al., 2016)). Production of acid and H_2O_2 with activity against red-complex bacteria has also been described (Lim et al., 2018).

A crucial indirect action mechanism of probiotics is the modulation of the immune responses of the host. These responses have been mostly studied in the gut, where probiotics have been proved to alter the balance of pro-inflammatory and anti-inflammatory cytokines secreted by epithelial cells (Stamatova and Meurman, 2009). As oral probiotics gained attention, studies have focused on their effect on immune regulation in the oral environment. Effects on immune responses range from reducing inflammatory factor production by oral pathogens to the up-regulation of the host's defence mechanisms (revised by Zhang et al. (2022)). Reductions in the production of pro-inflammatory factors such as tumour necrosis factor and interleukins (IL-1β, IL-6, IL-8, IL-17) by oral epithelial cells is one of the most recurrent responses described when assessing the effects of oral probiotics (Table 2). In some probiotic formulations, these decreases in pro-inflammatory factors have been linked to the production of arginine deiminase (Xie et al., 2007), an enzyme that can inhibit NO synthesis (Figure 1). Another interesting effect of probiotics is the reduction of pathogen-induced cytokine production and the antagonisation of cytokine-induced cell proliferation and apoptosis (Table 2). Regarding the effects on the hosts immune system, probiotics have been described to increase the production of anti-inflammatory factors such as interleukin 10 (IL-10) (Esteban-Fernández et al., 2019; Silva et al., 2022). The expression of β-defensins, the antimicrobial peptides that act at the epithelial level, is also increased by the action of some probiotic strains (Kobayashi et al., 2017). Additionally, increases in haemocyte density *in vivo* have been attributed to the use of lactobacilli-based probiotics (Santos et al., 2020). Attenuation of immune responses via the inhibition of nuclear factor kappa B (NF-κB) and the activation and inhibition of NO production in macrophages has also been described in response to probiotic uptake (Kim et al., 2020b).

Considering these action mechanisms just described, certain characteristics are interesting to find when looking for potential probiotic species. The most immediate characteristic related to the sustainability of the potential probiotic is the ability to adhere to oral surfaces successfully or to become integrated into preformed oral biofilms, competing for binding sites or co-aggregating with other microorganisms to remain viable in the mouth (Meurman, 2005). *A priori*, ideal probiotic candidates would be bacteria naturally occurring in the oral cavity, as they would be expected to better adapt and resist the oral environmental

conditions, adhere to soft or hard oral tissues/surfaces, and be integrated with the normal microbiota residing there (Meurman, 2005). Moreover, they should not entail a bacterial challenge for the immune system of the oral environment. Another desirable characteristic of a probiotic is the production of antimicrobial substances against harmful bacteria (Meurman, 2005). Once they have been selected based on their biological characteristics, probiotics must preserve their viability and functional properties after enduring the manufacturing processes necessary to administrate them. These processes include undergoing encapsulation, extrusion, lyophilization, and even processing of food products they may be integrated into.

Considering these characteristics, we will go over the most common genera used as probiotics in the oral field. Currently, it is found that probiotics with the potential to contribute to oral health are concentrated in the genera *Lactobacillus*, *Bifidobacterium, Weissella,* and *Streptococcus,* as well as certain scattered species like *Bacillus subtilis* and the yeast *Saccharomyces cerevisiae* (Zhang et al., 2022). The most common oral probiotics are members of the genus *Lactobacillus* (Meurman, 2005; Wade, 2021). They are classical gastrointestinal probiotics and common inhabitants of the mouth (Marsh et al., 2016; Philip et al., 2018), hence their translation to the oral field to assess their potential as oral probiotics is expected to be relatively simple. In addition, their antimicrobial activity has been extensively studied, especially in gastrointestinal probiotic applications (Meurman, 2005), and against oral yeasts (Meurman, 2005; Montero et al., 2019). Numerous trials have examined the effects of lactobacilli administration for oral health improvement, generally describing promising results, as inflammation and pathogen load are generally reduced in the presence of lactobacilli-derived probiotics (Tables 1 and 2). Several studies have tested the ability of probiotic lactobacilli to prevent caries (Montero et al., 2019) with successful results in the reduction of *S. mutans* counts, at least for the duration of the treatment time. Lactobacilli have also been tested for other oral diseases; a study on animal models with induced apical periodontitis has described a reduction in tissue inflammation following a lactobacilli-based probiotic administration (Cosme-Silva et al., 2020). These results open the door to further studies on the clinical practice of using probiotics as adjuvants to treat periodontitis. *Bifidobacterium* is another genus traditionally used as a gastrointestinal probiotic (Philip et al., 2018). Its application in the oral field followed that of lactobacilli, as *Bifidobacterium* can also be found in the oral cavity (Marsh et al., 2016). Administration of *Bifidobacterium*-containing probiotics lowered bacterial counts of several oral pathogens and regulated immune responses in *in vivo* studies and clinical trials

(revised in Zhang et al. (2022)). Some bifidobacteria-based probiotics have undergone clinical trials that demonstrated their ability to lower clinical signs of gingivitis (Kuru et al., 2017). Despite these promising results, it is worth noting that species of both the genus *Lactobacillus* and *Bifidobacterium* can be highly acidogenic and thus have the potential to initiate dental caries or aid in the progression of carious lesions (Meurman, 2005; Philip et al., 2018). This concern has been brought up when examining the efficacy of probiotics containing these genera and has prompted the search for alternatives in other groups.

Strains belonging to the genus *Weissella* have also been examined as oral probiotics. Members of this genus have been isolated from human saliva (Kang et al., 2011), although they are mainly found in fermented foods (Fusco et al., 2015). They comprise both potentially pathogenic and probiotic species. Probiotic strains of the species *Weissella cibaria* have been described to have antibiotic activities, as well as the ability to regulate immune responses (revised in Zhang et al. (2022)). Probiotic *W. cibaria* has a great co-aggregating capacity with oral pathogens (Kang et al., 2011, 2020; Kim et al., 2020a), which is thought not to interfere with pathogenic colonisation but suppress the growth of harmful bacteria.

Streptococci are part of the normal microbiota of the human mouth. They are primary colonisers, providing the necessary elements for a healthy ecological progression in the oral biofilm and the attachment of subsequent species (Marsh et al., 2016). However, some species of *Streptococcus* are potentially pathogenic to humans and have been linked to oral disease (Marsh et al., 2016). This is the case of *S. mutans* and caries development. In fact, plenty of probiotics are evaluated against *S. mutans* to assess their potential to treat and prevent caries (Montero et al., 2019). Nevertheless, among the genus *Streptococcus* we can also find strains with probiotic potential. This is the case, for example, of *Streptococcus dentisani*, a strain isolated from the oral cavity of caries-free individuals that has shown a great caries-protective capacity, thanks partially to its acid-buffering abilities (López-López et al., 2017). Probiotic streptococci can modulate periodontal pocket recolonisation, as their administration has been described to reduce and maintain low levels of anaerobic species and black-pigmented bacteria (Burton et al., 2006). This evidence points towards a possible application for periodontal disease management.

Finally, the emergence of studies on recombinant probiotics also offers interesting results in increasing efficacy against oral pathogens or minimizing problems associated with certain probiotics. This strategy is based on the genetic modification of existing probiotic strains

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so that they express pathogen-related proteins to stimulate pathogen recognition by the immune system (Marcotte et al., 2006; Ma et al., 2018). So far, a few in vivo studies point to the success of these modified probiotics (Marcotte et al., 2006; Ma et al., 2018). However, their applicability in humans is uncertain, as genetically modified organisms are not easily accepted as medical treatments.

The application of probiotics in the oral field for the prevention and treatment of dental caries and periodontal disease has been examined by individual studies and meta-analyses. There seems to be a tendency towards higher success rates in their application for periodontal disease control (Gruner et al., 2016), although meta-analyses find that probiotics do not always significantly improve oral health parameters related to the progression of these diseases (Donos et al., 2020). Several factors contribute to discrepancies found among studies, such as the specific probiotic strains or combination of strains used, the probiotic administration modes and schedules, and the dosage (Zhang et al., 2022). The general lack of information on the most effective doses and application modes for oral probiotics contributes to the variability of outcomes observed (Donos et al., 2020). As oral disease prognosis depends on the specific microbiota and immune system of each patient, probiotic therapy outcomes will also vary when administered to different patients (Zaura et al., 2017). Personalised treatments are already on the landscape of traditional oral treatments, and probiotic therapy should follow this trend (Zaura and Twetman, 2019). Preceding studies on intestinal probiotics have brought up the need for tailored probiotic treatments to achieve better colonisation rates and increase general effectiveness (Meurman, 2005). The objective of obtaining efficient, personalised probiotic treatments will probably arrive with the expansion of combinational therapies. Using different strains and even species combined in the same probiotic treatment could lead to synergistic effects, as dental plaque is an extraordinarily complex niche with multiple species inhabiting it. Genetic modification of probiotics could also be a successful approach to treating oral diseases (Marcotte et al., 2006; Ma et al., 2018) once the safety of these probiotics is proven for their use in humans.

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Table 1. Effects of probiotic bacteria on oral pathogens. Modified from Parga and Otero (in press).

Effect	Probiotic bacteria	Type of study			Proposed action mechanism	References
		In vitro	*In vivo*	Clin. trials		
Reduction of oral pathogen presence in oral tissues and exudates	Lactobacilli			✓	-	(Imran et al., 2015; Invernici et al., 2018; Shah et al., 2018)
	Bifidobacteria			✓	-	(Invernici et al., 2018)
	W. cibaria		✓	✓	-	*In vivo* (Kim et al., 2020a); clinical trials (Kang et al., 2020)
Change of the ratio between aerobic and anaerobic bacteria	Lactobacilli		✓		-	(Maekawa and Hajishengallis, 2014)
	Bifidobacteria		✓		-	(Oliveira et al., 2017)
Reduction of colonisation capacity of oral pathogens	Lactobacilli		✓		-	(Kobayashi et al., 2017)
	S. cristatus	✓			-	(Sliepen et al., 2008)
Inhibition of oral pathogen growth	Lactobacilli	✓			Release of postbiotics (lipase) (Jaffar et al., 2016)	(Zhao et al., 2012; Jaffar et al., 2016; Shah et al., 2018; Santos et al., 2020; Ding et al., 2021)
	Bifidobacteria	✓			-	(Invernici et al., 2020)
	S. dentisani	✓			-	(López-López et al., 2017)
	W. cibaria	✓			Production of acid, H_2O_2, and N-acetylmuramidase	(Lim et al., 2018)
Reduction of biofilm formation in oral pathogens	Lactobacilli	✓			Release of postbiotics (Ishikawa et al., 2021) and production of lipase (Jaffar et al., 2016)	(Jaffar et al., 2016; Shah et al., 2018; Ishikawa et al., 2021)
	Bifidobacteria	✓			-	(Argandoña Valdez et al., 2021)
	S. cristatus	✓			Production of arginine deiminase	(Xie et al., 2007)
Reduction of oral pathogens' adhesion to cells	Lactobacilli	✓			-	(Ding et al., 2021)
	Bifidobacteria	✓			-	(Invernici et al., 2020)
	S. dentisani	✓			-	(López-López et al., 2017)
	W. cibaria	✓			-	(Kang et al., 2011)
Down-regulation of virulence- and adhesion-related factors in oral pathogens	Lactobacilli	✓			-	(Ishikawa et al., 2020, 2021, 2021; Ding et al., 2021)
	S. cristatus	✓			Production of arginine deiminase	(Xie et al., 2007)
Change of cell wall structure of *P. gingivalis*	*S. dentisani*	✓			-	(López-López et al., 2017)
Induction of cell lysis in *F. nucleatum*	*S. dentisani*	✓			-	(López-López et al., 2017)

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Table 2. Effects of probiotic bacteria on immune response regulations and disease progression. Modified from Parga and Otero (in press).

Effect	Probiotic bacteria	Type of study			Proposed action mechanism	References
		In vitro	*In vivo*	Clin. trials		
Inhibit/reduce the expression of pro-inflammatory factors (interleukins, cytokines, interferon-gamma)	Lactobacilli	✓	✓	✓	Production of arginine deiminase (Maekawa and Hajishengallis, 2014)	*In vitro* (Kang et al., 2011; Zhao et al., 2012; Kobayashi et al., 2017); *in vivo* (Maekawa and Hajishengallis, 2014); clinical trials (Szkaradkiewicz et al., 2014)
	Bifidobacteria		✓	✓	-	In vivo (Oliveira et al., 2017; Silva et al., 2022); clinical trials (Kuru et al., 2017; Invernici et al., 2020)
	Streptococci	✓			-	(Zhang et al., 2008; Esteban-Fernández et al., 2019)
	W. cibaria	✓	✓		-	*In vitro* (Kang et al., 2011); *in vivo* (Kim et al., 2020a)
Increase the expression of anti-inflammatory factors	Bifidobacteria		✓		-	(Silva et al., 2022)
	S. dentisani	✓			-	(Esteban-Fernández et al., 2019)
Raise the expression of ß-defensin, TLR4, and CD4 in the gingiva	Bifidobacteria			✓	-	(Invernici et al., 2020)
Reduction of bone resorption and inflammation in apical periodontitis	Lactobacilli		✓		-	(Cosme-Silva et al., 2020)
Increase in haemocyte density upon *P. gingivalis* infection	Lactobacilli		✓		-	(Santos et al., 2020)
Decrease the expression of anti-inflammatory factors (IL-10)	*W. cibaria*		✓		-	(Kim et al., 2020a)
Reduce the number of $TRAP^+$ cells and infiltrating inflammatory cells	Lactobacilli		✓		-	(Gatej et al., 2018)
Raise the level of ß-defensin mRNA in gingiva, tongue, and saliva	Lactobacilli		✓		-	(Kobayashi et al., 2017)
Antagonises proliferation and apoptosis induced by *P. gingivalis*	Lactobacilli	✓			-	(Zhao et al., 2019)
Inhibit NF-kB activation and NO production in macrophages	*W. cibaria*	✓			-	(Kim et al., 2020b)

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3.3. Antimicrobial Peptides

Antimicrobial peptides (AMPs) constitute a natural defence mechanism against pathogenic microorganisms. The production of AMPs has been described in pluricellular organisms, where they can also be called host defence peptides, and bacteria. AMPs produced by the latter are commonly addressed as bacteriocins. Bacteriocins comprise several subclasses, from which the lantibiotics, produced by lactic acid bacteria, have been the most extensively studied (Rios et al., 2016). It has been demonstrated that human-produced AMPs specifically target microbes and contribute to the ecological equilibrium in the gut (Wade, 2021). Bacteriocins have an additional role in providing ecological advantages derived from the elimination of competitor bacteria and the subsequent increase in available nutrients in their surroundings (Rios et al., 2016).

AMPs are short, often post-translationally modified polypeptides consisting of 12–50 amino acids synthesized via the ribosomal way. These peptides have varied structures that condition their mode of action, and they are ubiquitous in nature. The most common bactericidal mechanism of AMPs is the formation of pores in microbial membranes, although the targeting of intracellular components has also been proposed as responsible for the killing effects of AMPs (Rios et al., 2016; Mai et al., 2017). The affinity of AMPs for microbial membranes is due to their physicochemical characteristics: the AMPs are predominantly positively charged due to the presence of amino acids such as cysteine and lysine, and bacterial membranes tend to be negatively charged due to the presence of hydroxylated phospholipids (gram-negative bacteria) and teichoic acids (gram-positive bacteria) (Brogden, 2005). Contrarily, mammalian cells tend to be more neutral in terms of charge, and thus AMPs are much less likely to attach to them (Mai et al., 2017). This selectivity makes them interesting as targeted therapeutics against pathogens, as they do not present high toxicity towards host cells (Rios et al., 2016; Mai et al., 2017). After the initial attraction towards the microbial membranes, AMPs can be integrated into them or go through them to reach the bacterial cytoplasm (Mai et al., 2017). AMPs tend to have an amphipathic structure that allows them to interact with microbial components and other innate defence molecules (Marsh et al., 2016; Rios et al., 2016). AMPs are generally constituted by a C-terminal domain, responsible for pore formation in bacterial membranes, connected to an N-terminal domain, responsible for the binding to bacterial surface receptors. The integration of AMPs in the microbial membranes can disrupt the membrane

structure, eventually causing cellular death. On the other hand, the permeabilization of the membrane allows for AMP leakage into the cytoplasm, where it can bind to nucleic acids and cellular components to disrupt the normal functioning of the microbial metabolism (Mai et al., 2017; Chassaing and Cascales, 2018).

The production of human AMPs has been described in the oral cavity in salivary glands, oral mucosa, and dental pulp. Some AMPs have also been found in gingival crevicular fluid (GCF) (Marsh et al., 2016; Niu et al., 2021). There are three main types of AMPs described in the oral context. Defensins can be expressed constitutively in salivary glands or upon a bacterial-induced inflammatory signal (Marsh et al., 2016) and exert antimicrobial activity against bacteria, fungi, and viruses (Marsh et al., 2016; Niu et al., 2021). Human β-defensins (hBDs) can be released by the host's immune cells and have been detected in numerous oral mucosal surfaces and epithelia (Marsh et al., 2016). Another type of AMPs found in human salivary glands are histatins. Histatins are cationic, histidine-rich peptides with antibacterial and antifungal activities. They are active against proteases and adhesins of host and microbial origin and can also inhibit bacterial-induced cytokine production by binding to pathogenic outer membrane proteins (Marsh et al., 2016). Lastly, the LL-37 peptide is the only member of the cathelicidins that has been described in the oral environment in humans. LL-37 is produced in both epithelial cells and neutrophils and has antibacterial properties (Marsh et al., 2016; Mai et al., 2017). In addition, the LL-37 peptide has immunomodulatory activities, as it can bind to inflammatory molecules of microbial origin and attract immune cells to the infection site (Marsh et al., 2016).

Bacteriocin production by human oral bacteria is rather common, with members of the genera *Streptococcus, Lactobacillus, Fusobacterium,* and *Prevotella* presenting this activity (Barbour et al., 2022). Thus, many of these groups are considered to have probiotic potential, as we have revised in section 2.2 of this chapter. Most of the studies on isolated bacteriocins for therapeutic approaches have been performed with the subclass lantibiotics (Mathur et al., 2018).

AMPs have been presented as alternatives to antibiotics to treat microbial infections in humans for their selectivity towards microbial cells and less expected tendency to induce resistance. The AMPs accepted/approved by the FDA and already on the market are polymyxins B and E (colistin) and daptomycin (Rios et al., 2016). Other natural AMPs have been tested as therapeutics *in vitro*, and some are currently undergoing *in vivo* assays and clinical trials

(Devocelle, 2012; Rios et al., 2016). These efforts have focused on different infections, such as respiratory infections or medical devices-related infections. These investigations have brought to light some disadvantages of the use of natural AMPs, such as certain unspecific activity towards mammalian cells, which has been reported especially with defensins (Yeaman and Yount, 2003). Some characteristics of natural AMPs may reduce their efficacy when used in the oral context, such as their susceptibility to protease action and subsequent loss of antimicrobial activity (Rios et al., 2016). Fortunately, advances in the modification of peptide sequences have made possible the creation of synthetic or "targeted" antimicrobial peptides. These synthetic peptides possess advantageous characteristics regarding their stability, bioavailability, selectivity, and half-life (Devocelle, 2012; Bechinger and Gorr, 2017; Guo and Edlund, 2017).

Both natural and synthetic AMPs have been investigated for the treatment of oral diseases, both for their direct effects on microbes and their immunomodulatory properties. Several formulations containing AMPs are already commercialized for topical and mucosal infections outside the oral context (Rios et al., 2016). However, in the oral field, most studies have been performed *in vitro* and have not yet reached clinical trials.

Natural AMPs have been linked to oral health, as they have been found in significantly higher proportions in the metagenomes of caries-free individuals (Belda-Ferre et al., 2012). Moreover, the distribution of strains with low susceptibility to AMPs has been correlated with caries progression (Goeke et al., 2018). Studies on natural AMPs point to a protective effect on oral health, acting against cariogenic pathogens (Niu et al., 2021). On the other hand, synthetic AMPs against cariogenic species are mostly studied *in vitro* and mainly on planktonic cells, although reports of antibiofilm activities can also be found (revised in Niu et al. (2021)). Two derivates of human β-defensin-3 have proved their inhibitory activity on *in vitro* streptococcal biofilm formation (Kreling et al., 2016; Ahn et al., 2017). KSL-W, a synthetic peptide, has shown great stability *in vitro* in saliva, conserving its antimicrobial activity in this environment (Na et al., 2007). When developing a formulation that included KSL-W and cetylpyridinium chloride (CPC) for caries control, these two components displayed synergistic effects disrupting *in vitro* oral biofilms formed on hydroxyapatite (HA) discs (Leung et al., 2009). Recently, the oral bioavailability of KSL-W has been improved by fusing this AMP with a peptide that binds to HA (Huang et al., 2016). Another example of an AMP targeting a cariogenic pathogen is the synthetic peptide C16G2. C16G2 was engineered to improve its stability and antimicrobial

abilities, and it has been proven to specifically target *S. mutans* in *in vitro* saliva-derived biofilms without affecting closely related non-cariogenic streptococci (Eckert et al., 2006; Guo and Edlund, 2017). Different formulations of this synthetic AMP have been tested in clinical trials for oral health purposes, with C16G2-based varnishes, mouth rinses, oral gels, and strips reaching phase II of clinical trials (identifiers: NCT03196219, NCT02044081, NCT02254993, NCT02509845, NCT02594254, NCT03004365 and NCT03052842, available in the ClinicalTrials.gov database of the U.S. National Library of Medicine (2022)). To the best of our knowledge, no further results have been made available on the progression of said trials.

Bacteriocins have also been tested *in vitro* as alternatives to antibiotics on cariogenic species. The lantibiotic nisin, for example, has been successfully tested *in vitro* against monospecific biofilms of *S. mutans* (Mathur et al., 2018). However, when nisin was examined against a three-species biofilm containing *S. oralis, S. gordonii*, and *A. naeslundii*, no antibiofilm activity was observed, and antimicrobial action was moderate (Corbin et al., 2011). Interesting results have been obtained by Arslan et al. (2009) using the iron-free form of lactoferrin on dual-species biofilms formed onto saliva-coated surfaces. Lactoferrin was able to significantly reduce the biofilm of *S. gordonii-F. nucleatum,* and of *S. gordonii-P. gingivalis* (Arslan et al., 2009). Also, the peptide 1018 almost completely eliminated the biofilm formation of supragingival plaque samples cultured *in vitro* (Wang et al., 2015). This evidence points towards a promising application of bacteriocins to control cariogenic biofilms.

Some bacteriocins may also potentially prevent periodontal disease-related microorganisms from attaching and further developing into a biofilm. Nisin, which action was already commented against oral streptococci, was shown to inhibit the growth of both early and late colonisers in saliva-derived biofilms formed *in vitro* (Shin et al., 2015). Among the late colonisers assessed, *P. gingivalis, Prevotella intermedia, Treponema denticola,* and *Aggregatibacter actinomycetemcomitans* were the most sensitive to nisin. In addition, multispecies biofilms were reduced in terms of biomass and thickness when cultured in the presence of nisin (Shin et al., 2015). Interestingly, the nisin effect on oral epithelial cells was assessed *in vitro*, showing no toxicity when used at concentrations showing antimicrobial and antibiofilm activities (Shin et al., 2015). A hallmark of periodontal diseases is the inflammation that is common to all of them. The potential of AMPs as a strategy against these dysbiotic states resides partly in their immunomodulatory activities. Lactoferrin, already proposed for caries prevention, has also shown very interesting anti-inflammatory effects, attributed to its ability to

sequester bacterial LPS (Berlutti et al., 2012). Lactoferrin is, in fact, a component of several products destinated for oral health maintenance, with beneficial effects being demonstrated in several clinical trials (Butera et al., 2021, 2022).

Regarding oral candidiasis, AMPs have also been proposed as therapeutics, as many natural AMPs have antifungal activity. For example, histatin 5, an AMP naturally present in human saliva, has demonstrated antifungal activity against *Candida*. In particular, Rothstein et al. (2001) established that it was just a 12-amino acid fragment of this histatin that contained the antifungal activity, as they proved *in vitro* against several *Candida* spp. clinical isolates (Rothstein et al., 2001). *In vitro Candida* biofilms formed onto the materials from which dental devices are made, was also inhibited by histatin 5, with efficient results upon prolonged exposure (Pusateri et al., 2009). Delivery of histatin 5 in gels for topical oral application has been tested *in vivo* in animal models, displaying protective effects against *Candida* infection and health restorative effects against pre-existing lesions (Kong et al., 2016). These findings support the feasibility of histatin 5 as a novel therapeutic agent. More *in vivo* assays and clinical trials are needed to further validate these approaches for oral health maintenance and their application in patients at risk of oral candidiasis.

The application of AMPs to oral health relies on previous knowledge obtained from the treatment of other infections. This fact paves the way in multiple aspects of the investigations related to them, for example, their potential to be engineered to obtain improved features or their interactions with other therapeutic compounds, such as antibiotics. This combinational antibiotherapy has been successfully applied outside the oral field (Rios et al., 2016), and therefore, it is expected to be also tested for oral infections and diseases. As we have shown throughout this section, currently, the main limitation is the scarce evidence of the effectiveness of AMPs *in vivo* and in clinical trials, which will probably be overcome in the upcoming years, as several compounds are entering the pipeline for these procedures.

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3.4. Phage Therapy

Bacteriophages or phages are viruses with the capacity to infect and kill bacterial cells. Due to their specificity and self-replicating nature, the use of bacteriophages for the treatment of certain multi-drug resistant bacterial infections, a strategy also known as phage therapy, is gaining increasing attention (Rios et al., 2016; Ghosh et al., 2019), and its efficacy has been already tested in the clinical practice (Dedrick et al., 2019). The existence of phages in the oral environment has been known for decades, and they have been found in oral samples, including phages of important oral pathogens, such as *F. nucleatum* and *S. mutans* (Machuca et al., 2010; Dalmasso et al., 2015; Baker et al., 2017; Szafrański et al., 2017, 2021; Fernandes et al., 2018; Steier et al., 2019). As in many other microbial communities, oral phages are thought to play a significant role in shaping the oral microbiome by influencing the species equilibrium and acting as mediators of genetic exchange (Baker et al., 2017), but the ecological involvement of bacteriophages in oral diseases has not been completely clarified yet. As HTS techniques have been implemented for oral studies, a broader picture has been constructed, and putative phages of numerous genera belonging to the major bacterial phyla of the oral cavity have been described (Pride et al., 2012). In fact, a significantly higher abundance of lytic phages was found in the subgingival plaque of periodontitis subjects compared with healthy subjects, suggesting that phages play a critical role in modulating the microbial community and contributing to periodontal disease progress (Ly et al., 2014). Transcriptome analysis of the oral bacteriophage community performed on healthy donors and periodontal disease patients hinted at a more active role of lytic phage molecules in the latter (Santiago-Rodriguez et al., 2015). However, more studies are needed to establish the relevance of viral gene expression on oral disease onset and progression.

Bacteriophages can have two life cycles: a lytic cycle in which phage infection leads to the death of the bacterial host, and a lysogenic cycle in which viral DNA is integrated into the bacterial chromosome, replicating "silently" within the host without killing it. Lytic bacteriophages are the most suitable for developing phage-based treatments, as lysogens (bacteria containing virus with a lysogenic cycle) present increased pathogenicity and elevated resistance to phage therapy (Fernandes et al., 2018). Interest in the use of phage therapy resides in the bactericidal activity of the bacteriophages but also in the possibility of using them as "Trojan horses" to deliver genes encoding for enzymes or toxins to the bacteria (Philip et al., 2018). The ultimate purpose is for these genes to be translated by the host bacteria, ultimately

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inflicting damage on it. These enzyme- or toxin-encoding genes can either be naturally present in the genome of the bacteriophage or be genetically engineered. Additionally, isolated phage enzymes, such as the endolysins used to digest the bacterial walls and spread to neighbour cells, have been explored as an antimicrobial strategy (Rios et al., 2016) and have also been proposed as a viable strategy *in vitro* against oral pathogens (Steier et al., 2019).

As stated previously, it is common to find bacteriophages in the oral microbiome (Pride et al., 2012; Szafrański et al., 2017, 2021), including those with activity against bacterial genera implicated in oral health and disease. Several studies have been conducted on bacteriophages' use to restore the oral microbial equilibrium. Despite this, nowadays, investigations in oral phage therapy are mostly limited to *in vitro* studies, with few exceptions. For example, *Enterococcus faecalis* is one of the species in which more bacteriophages have been described, and a wide range of phages are available for the control of this species in the oral context (revised in Szafrański et al. (2017)). The implication of *E. faecalis* in infections after root canal treatment and the incidence of multiresistant strains in this species has promoted the studies on alternative therapies against this bacterium. Different bacteriophages have successfully inhibited *E. faecalis* growth *ex vivo* in root canals (Paisano et al., 2004; Khalifa et al., 2015). It is also interesting to assess the effect of phage therapy in biofilms, as these structures are linked to oral disease progression. There are a few reports on inhibiting *E. faecalis* biofilm formation using bacteriophages in *in vitro* experiments (Khalifa et al., 2015; Bhardwaj et al., 2020) and *ex vivo* in root canals (Khalifa et al., 2015). To the best of our knowledge, the only *in vivo* study on phage therapy against oral pathogens is the work of Xiang et al. (2022) on the effect of a bacteriophage against an *E. faecalis* strain isolated from a periapical periodontitis lesion. Confirming the results of *in vitro* and *ex vivo* assays, an abdominal infection model and a periapical periodontitis model, both performed in rats, showed that treatment with the bacteriophage successfully decreased mortality and completely prevented periapical periodontitis, respectively (Xiang et al., 2022). Phage therapy was even more effective than antibiotics tested in the same conditions (Xiang et al., 2022). Thus, these results open the door to further *in vivo* studies to assess the feasibility of using phage therapy after root canal treatment as a prevention strategy and on ongoing infections as a treatment approach.

Mutans and mitis streptococci are common targets for bacteriophages, with a high number of phages active against *S. mutans*, *S. mitis, Streptococcus sobrinus*, and *Streptococcus oralis* (revised in Szafrański et al. (2017)). Studies report higher lytic activity in bacteriophages

targeting mutans streptococci than mitis streptococci (Szafrański et al., 2017). Independent studies have found different *S. mutans* bacteriophages that can almost completely inhibit biofilm formation *in vitro* in this species (Ben-Zaken et al., 2021; Rajabi et al., 2021). Interestingly, several modified lytic enzymes obtained from streptococcal phages have been patented for oral health maintenance (Delisle, 1996; Fischetti and Loomis, 2004; García et al., 2014). Additional *in vitro* studies on enzymes against streptococci described a lysin that was selectively active against 3 different streptococci; *Streptococcus pneumoniae, S. oralis,* and *S. mitis* (van der Ploeg, 2010). Phage lysins appear as a promising new antimicrobial strategy that can also synergically act with antibiotics by increasing cell wall permeability and decreasing the antibiotic minimum active concentration (Rios et al., 2016). Further *in vivo* and subsequent safety and clinical trials await before this strategy can be further implemented.

The evidence on phage therapy against other oral bacteria is mostly limited to *in vitro* studies with moderately successful results. Briefly, additional bacterial taxa with reported bacteriophages comprise members of the genera *Aggregatibacter, Fusobacterium, Lactobacillus, Prevotella, Treponema,* and *Veillonella* (revised in Szafrański et al. (2017) and Steier et al. (2019)). Among these, a limited number of species or even strains have been described to have specific bacteriophages (Szafrański et al., 2017; Steier et al., 2019).

Phage therapy is a promising alternative or adjuvant to antibiotics (Tagliaferri et al., 2019), especially for root canal post-intervention (Fernandes et al., 2018). The advantages of phage therapy include a low impact on the commensal microbiota, easy isolation and purification processes, the possibility to genetically engineer phages, and their increased efficacy on biofilms compared with conventional antimicrobial agents, as reported *in vivo* against *E. faecalis* (Xiang et al., 2022). Nevertheless, there are also some limitations associated with their use. Their high strain-specificity calls for precise diagnostic procedures or, when these are not available, for the use of phage cocktails. Their potential immunogenicity and the risk of lysogeny have also been brought up as a general concern (Abedon et al., 2011; Szafrański et al., 2017; Ghosh et al., 2019). All these aspects call for further investigations regarding the safety and applicability of this strategy. To date, the lack of clinical trials and regulations regarding their use limits the validation of phage therapy as a strategy to treat and prevent oral disease.

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3.5. Quorum Sensing Inhibition

3.5.1. Quorum Sensing

Bacteria use complex communication systems to coordinate gene expression in a cell density-dependent manner, a process known as Quorum Sensing (QS) (Figure 2). This phenomenon relies on the accumulation of small signalling molecules or autoinducers (AIs) produced by bacteria and released to the extracellular milieu. The signalling molecules bind to specific receptors, allowing bacteria to sense the surrounding cell density. Once the concentration of signalling molecules reaches a certain threshold, i.e., a *quorum*, the intracellular signalling cascades are activated and the bacterial population regulates its gene expression in a synchronized manner (Fuqua et al., 1994). This regulated response must extend beyond physiological changes required to metabolise the signalling molecule, for it to be considered as a QS communication process (Winzer et al., 2002b). Besides high cellular density, environmental conditions also influence QS signal production and stability, conditioning the availability of these molecules to bind to their receptors (Yates et al., 2002; Kalia, 2014; Mayer et al., 2018).

QS phenomena are involved in the regulation of numerous genes, which can be clustered into four functional categories (Grandclément et al., 2016); cell maintenance (enzyme production, acid resistance, sporulation, synthesis of siderophores), cell behaviour (biofilm formation, adhesion, motility), horizontal gene transfer (regulation of plasmid conjugation, competence), and host-microbe and microbe-microbe interactions (production of virulence factors, EPS, antibiotic compounds, host colonisation factors). By regulating these functions at high cell density levels, bacteria optimise their energetic resources and increase their fitness. As many relevant pathogenic bacteria regulate their biofilm formation through QS, these communication pathways have been proposed as potential targets for developing strategies to inhibit oral biofilm formation (Muras et al., 2018, 2020b; Muras and Otero, 2020). Furthermore, because strategies targeting QS do not interfere with bacterial growth, these approaches have been proposed to be less likely to induce bacterial resistance (Romero et al., 2014, 2015; Mayer et al., 2015, 2018; García-Contreras et al., 2016; Guendouze et al., 2017; Muras et al., 2018).

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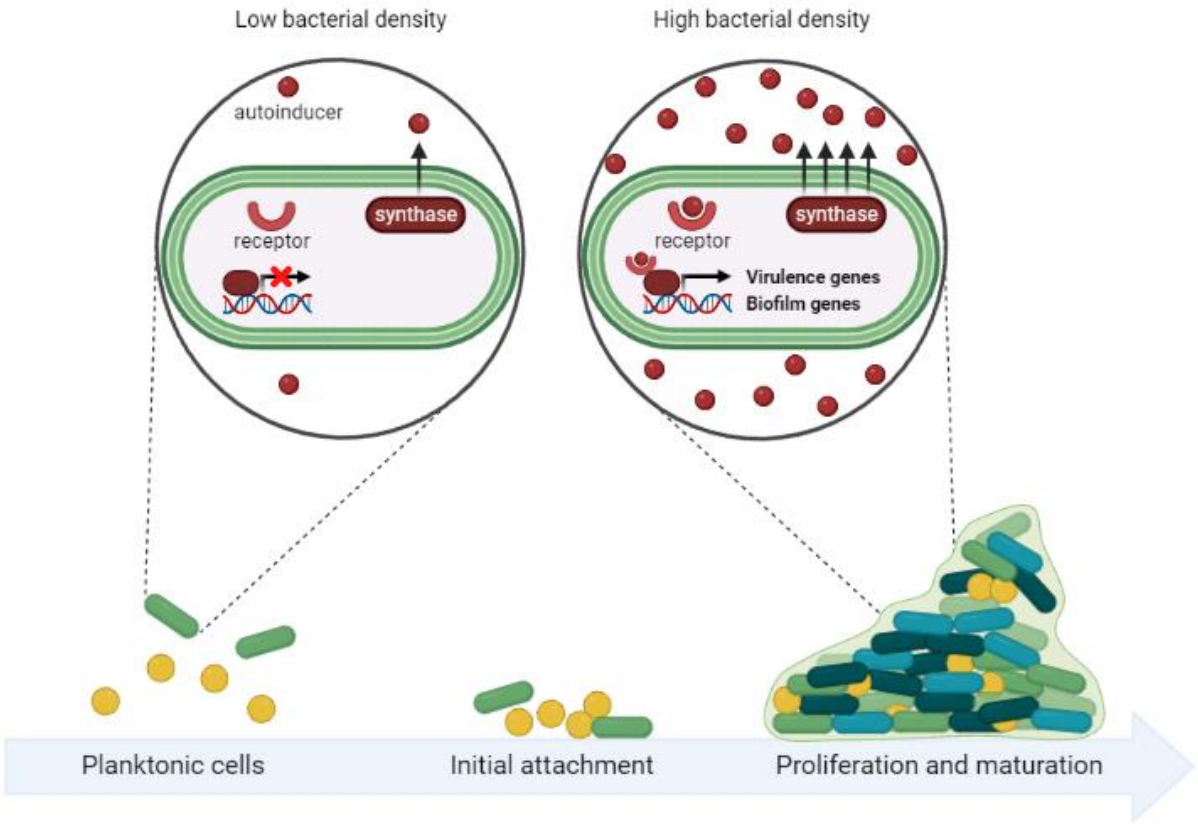


Figure 2. Quorum Sensing-dependent regulation of gene expression of biofilm-related and virulence-related genes. Modified from Muras et al. (2022).

There are several QS systems described in bacteria, mediated by different QS signalling molecules. The existence of species-specific systems and of systems shared by different taxa, allows intra-specific and inter-specific communication among bacteria, and a broader assessment of the surrounding bacterial populations (Schauder and Bassler, 2001). The best-studied QS signalling molecules, are the universal autoinducer-2 (AI-2), the autoinducer peptides (AIPs), and the *N*-acyl-homoserine lactones (AHLs) (Figure 3). The relevance in the oral context of AI-2, produced by both gram-positive and gram-negative bacteria, and the AIPs of gram-positive bacteria, is conventionally accepted (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Kolenbrander et al., 2006). However, AHLs have been excluded from the current paradigm of QS in dental plaque (Frias et al., 2001), even though multiple pieces of evidence suggest an important role of these signals in the oral cavity (Muras et al., 2022). Prevention of bacterial coaggregation and reduction of dental plaque are supposed to have beneficial effects on oral health. Thus, understanding the QS processes that are active inside oral biofilms is crucial to developing new strategies for preventing oral diseases.

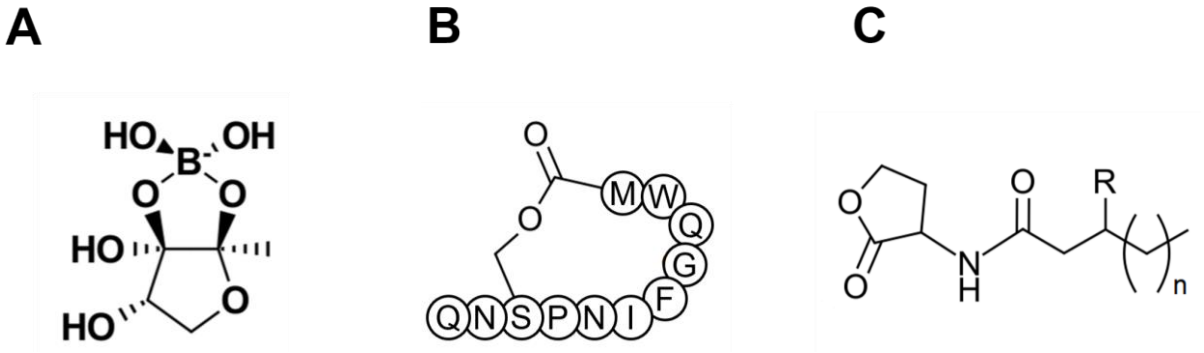


Figure 3. Main QS signals. A. Autoinducer-2 (AI-2) of *Vibrio harveyi*. B. Autoinducer peptide of *Enterococcus faecalis*. C. General structure of an *N*-acyl-homoserine lactone (AHL).

- **Autoinducer-2**

The presence of AI-2 has been described in both gram-negative and gram-positive taxa, and this family of autoinducers has been considered as an "universal" or interspecific QS signalling system (Miller and Bassler, 2001; Federle and Bassler, 2003). The differential responses to intraspecific and interspecific signalling molecules would allow bacteria to modulate its behaviour depending on whether they dominate or not a given population (Schauder and Bassler, 2001).

AI-2 designates a family of cyclic compounds derived from 4,5-dihydroxy-2,3-pentanedione (DPD) by the enzymatic action of LuxS (Winzer et al., 2002a; Williams et al., 2007). AI-2 compounds are highly variable in structure and have been found in several Pseudomonadota, Bacillota (former Firmicutes), and Actinomycetota (former Actinobacteria), being the most common signalling molecule identified to date (Pereira et al., 2013). Nevertheless, the role of AI-2 as a QS signalling molecule has been disputed, as it is a by-product of the detoxification of S-adenosylhomocysteine (SAH) in the activated methyl cycle. LuxS is one of the enzymes that can remove SAH and generate homocysteine. While performing this reaction, it generates DPD, which can spontaneously cyclise into different furanone moieties, which different bacteria recognise as their active AI-2 signals (Figure 3A) (Williams et al., 2007; Papenfort and Bassler, 2016). Therefore, due to the role in central metabolism of LuxS, its implication in QS should be confirmed in a case-by-case manner through the description of a specific receptor, and not solely through the observation of phenotypical modifications upon LuxS mutation.

The production of AI-2 has been described in pure cultures of the oral pathogens *F. nucleatum, P. gingivalis,* and *Prevotella intermedia* (Frias et al., 2001). Moreover, AI-2-mediated QS has been pinpointed as critical during biofilm development (Frias et al., 2001; Burgess et al., 2002; Kolenbrander et al., 2006; Rickard et al., 2006). AI-2 has been described to regulate biofilm formation in the periodontopathogen *A. actinomycetemcomitans* (Novak et al., 2010), and to be essential for mutualistic *A. naeslundii* and *Streptococcus oralis* biofilm (Rickard et al., 2006). In addition, AI-2 obtained from *F. nucleatum* induces an increase in biofilm mass in mixed cultures of the red complex bacteria *P. gingivalis, T. denticola* and *T. forsythia* (Jang et al., 2013). Its production and detection is higher in pathogenic species than in commensal bacteria, and thus AI-2 communication has been proposed as a starting point for the establishment of dysbiosis in the oral environment, inducing the proliferation of pathogenic bacteria and eventually leading to a pathogenic periodontal biofilm (Frias et al., 2001; Kolenbrander et al., 2006; Rickard et al., 2006).

- **Autoinducer peptides**

Gram-positive bacteria use oligopeptides as autoinducers for communication purposes. These autoinducer peptides (AIPs) are actively exported to the extracellular milieu and it is common that during this transport, they are modified to adopt their active structure, which can comprise lanthionines, thiolactone rings and lactone rings (Booth et al., 1996; Mayville et al., 1999; Nakayama et al., 2001) (Figure 3B). AIPs interact with histidine kinase sensors bound to the membrane, activating a phosphorylation cascade that ultimately activates a response regulator, modulating gene expression. AIPs differ between taxa, and sometimes even between groups within the same species. The cognate receptors are highly sensitive to the AIPs' structure, allowing bacteria an extremely precise recognition of the surrounding population (Waters and Bassler, 2005). One example of this is the QS system of *Staphylococcus aureus*; the accessory gene regulator or Agr. The Agr regulon controls the shift from the expression of an adhesion phenotype at low cell densities, to a virulent and disseminating phenotype at high cell densities (Lyon and Novick, 2004). The Agr QS system relies on an autoinducer peptide encoded by *agrD,* and the two-component sensor kinase (AgrC) and response regulator (AgrA) (Ji et al., 1995). The AIP is exported by AgrB, which is membrane-bound and modifies the AIP during its transport, adding a thiolactone ring that renders the molecule functional (Saenz et al., 2000). Binding of the AIP to AgrC activates the phosphorylation cascade and ultimately results

in the AgrA-mediated induction of gene expression. Besides the control of genes related to biofilm dispersal and virulence, activation of the Agr system induces the expression of the *agrBDCA* operon, entering a positive feedback loop that results in the increase of extracellular AIP levels and drives the whole bacterial population to a "quorum" state (Lyon and Novick, 2004). As mentioned before, recognition of AIPs by their cognate receptors is highly structure-specific. This has led to the classification of *S. aureus* in four categories, dependent on the sequence of the AIP they use. The specificity of AIP recognition is such, that in the presence of the other groups of *S. aureus*, AIPs are able to bound to their non-cognate AgrC receptors and inactivate the phosphorylation cascade (Lyon et al., 2002). This confers an ecological advantage to the *S. aureus* group that first establishes its QS system in a mixed population.

The Agr system has homologues in other gram-positive taxa, such as the Fsr system of *Enterococcus faecalis*, a species linked with caries and root canal infections. In this species, genes under the control of the Fsr QS system have been linked both with adhesion and biofilm formation, and with the expression of virulent phenotypes and dispersal (Bourgogne et al., 2006; Pinkston et al., 2011; Ali et al., 2017; García-Solache and Rice, 2019). Similarly to the Agr system in *S. aureus*, the Fsr system comprises an AIP, the gelatinase-biosynthesis activating pheromone (GBAP), exported and modified by FsrB to finally present a lactone ring in its structure (Figure 3B). GBAP binds to a histidine kinase sensor, FsrC, and activates the response regulator FsrA. This system is also highly specific, and several molecules that share structural analogies with GBAP have been demonstrated to inhibit Fsr-controlled responses (Nakayama et al., 2009, 2013; Desouky et al., 2015).

In oral streptococci, the competence-regulatory system is controlled by cell-density mechanisms via the production and recognition of the competence-stimulating peptide (CSP) (Li et al., 2001, 2002). The competence system of streptococci comprises a two-component transduction system, ComD/ComE, different from the one found in staphylococci and enterococci, in which CSP binds to a histidine kinase sensor and induces the phosphorylation of response regulator proteins, ultimately regulating gene expression. CSP-controlled phenotypes range from bacteriocin production, to regulation of autolysis, and biofilm formation in several streptococcal species (Li et al., 2002; Petersen et al., 2004; Senadheera and Cvitkovitch, 2008).

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- ***N*-acyl-homoserine lactones**

N-acyl-homoserine lactones (AHLs) are QS signalling molecules typically used by gram-negative bacteria. Their structure comprises a homoserine lactone (HSL) ring linked by an amide bond to an acyl chain with 4 – 20 carbon atoms, which renders them hydrophobic and facilitates diffusion through gram-negative bacteria outer membranes (Fuqua and Greenberg, 2002; Arashida et al., 2018). The acyl chain can present a carbonyl group (oxo-substituted AHLs) or a hydroxyl group (hydroxy-substituted AHLs) in the third carbon (Figure 3C). Moreover, less common AHLs with diverse modifications and even unsaturations in their acyl chains have been described (Laue et al., 2000; Schaefer et al., 2008; Doberva et al., 2017). Together with the acyl chain length, the different possible substitutions condition the specificity of the AHL, i.e., its recognition by the AHL receptor (Waters and Bassler, 2005). It has also been proposed that AHLs with longer acyl chains may be actively transported through bacterial membranes (Pearson et al., 1999). Based on their structure, AHLs are typically abbreviated by indicating the oxidation state of the third carbon in the acyl chain, herein represented as "O-" for oxo-substitutions, "H-" for hydroxy-substitutions, or no prefix for unsubstituted AHLs, followed by the number of carbons in the acyl chain and the abbreviation HSL (e.g., C_4-HSL for butyryl-homoserine lactone). This is the nomenclature that will be used throughout this thesis.

The production of AHLs is typically regulated by a LuxI/LuxR system. LuxI is the AHL synthase that derives the lactone moiety from S-adenosylmethionine (SAM) and an acyl chain obtained from fatty acid biosynthesis pathways. Upon entering the bacterial cytoplasm, AHLs bind to the hydrophobic acyl-binding pocket of LuxR, the response regulator. The AHL-LuxR complex acts as the transcriptional regulator that mediates the activation of gene expression (Decho et al., 2010; Papenfort and Bassler, 2016). The LuxI/LuxR system is the most extended among gram-negative bacteria, but it is not the only one that has been described in these taxa. One of these alternative synthases is LuxM, present in *Vibrio harveyi,* and in *Aliivibrio fischeri,* where it receives the name of AinS. Although the LuxM synthase is not homologous with LuxI, they use the same precursors for AHL production. Another difference with the LuxI/LuxR system is that LuxM and AinS are associated with sensor kinases (termed LuxN and AinR, respectively), which activate an intracellular phosphorylation cascade to regulate gene expression (Fuqua and Greenberg, 2002). In addition, a third class of AHL synthases, termed HdtS, has been described in *Pseudomonas fluorescens* (Laue et al., 2000). HdtS does not belong

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to the LuxI or LuxM synthase families, and it has been demonstrated to produce at least three different AHLs (C_4-HSL, C_6-HSL, and $OC_{14:1}$-HSL) (Laue et al., 2000).

In the last decade several molecules different than AHLs and able to bind to LuxR-type receptors have been described. These molecules are termed photopyrones (PPY), dialkylresorcinols (DAR), and *p*-coumaroyl-homoserine lactone, which synthases are PpyS, DarABC, and RpaI, respectively (Brameyer et al., 2015; Tobias et al., 2020). Another exception to the standard AHL-mediated QS systems are bacteria that do not possess a known AHL synthase, but that have LuxR or LuxR-like receptors that can sense AHLs produced by other bacteria. This receptors are known as LuxR solos (Tobias et al., 2020). The most studied example is the LuxR-like receptor SdiA, present in *Escherichia coli* and closely related taxa, such as *Salmonella* (Michael et al., 2001). This receptor has been later characterised as promiscuous, recognising molecules other than AHLs, but structurally related to these molecules, as agonist ligands (Styles and Blackwell, 2018).

AHL production has been identified in numerous genera of Pseudomonadota (former Proteobacteria), and more recently in taxa outside of this group, even in a gram-positive representative, which to date constitutes the only report on AHL-production in gram-positives (Williams et al., 2007; Romero et al., 2010; Biswa and Doble, 2013). AHLs have been traditionally excluded from the paradigm of cell-cell communication in the oral cavity due to the lack of success in detecting their production by the main oral pathogenic species in pure cultures (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Kolenbrander et al., 2006). Recently, AHL production has been described by bacteria isolated from caries lesions (Goh et al., 2014, 2016) and human tongue surface (Yin et al., 2012b, 2012a; Chen et al., 2013). Additionally, the production of an AHL has been described in pure cultures of *P. gingivalis* (Muras et al., 2020b). *P. gingivalis* has also been shown to respond to AHLs and AHL analogues by changing its protein expression profile, slowing down its growth, and reducing biofilm formation (Komiya-Ito et al., 2006; Asahi et al., 2010). This behaviour suggests that *P. gingivalis* may have a solo LuxR receptor, the presence of which has been yet-undescribed. Another study that linked AHLs and oral pathogens was conducted by Muras et al. (2020a), who exposed an *in vitro* multispecies biofilm originated from saliva samples to AHLs, describing a change in protease activity and lactic acid production in the presence of these molecules. Moreover, the presence of AHLs increased the proportion of the orange complex bacteria *Peptostreptococcus* and *Prevotella* (Muras et al., 2020a). In the light of this evidence,

the role of AHLs in QS communication in the oral environment should be revised (Muras and Otero, 2020; Muras et al., 2020b, 2022).

3.5.2. Quorum Sensing Inhibition and Quorum Quenching

Given the importance of QS systems in biofilm development, these processes have been proposed as a target for the control of oral biofilm formation. The interference with QS, also known as Quorum Sensing Inhibition (QSI), was firstly described in the marine algae *Delisea pulchra*, that inhibits bacterial biofilm formation on its surface due to the production of furanone compounds structurally similar to AHLs (Givskov et al., 1996). Later, it has been demonstrated that bacteria have also developed QSI systems, probably as a competitive strategy against other bacteria (Weiland-Bräuer et al., 2015).

QSI systems are widely diverse, with inhibitors of varied structure and function, capable of exerting their effect on different steps of the QS response pathways (Romero et al., 2011; Grandclément et al., 2016). QSI can be achieved through the production of antagonist molecules, or through enzymatic inactivation of the QS signalling molecules, termed Quorum Quenching (QQ) (Dong et al., 2001). As QSI does not affect bacterial survival abilities in the vast majority of cases, these strategies would not exert selective pressures on the bacterial population and would not lead to the rapid insurgence of resistance to the treatment (Romero et al., 2015). Hence, there is a growing interest in the search for treatments based on QSI or QQ to replace or complement antibiotic therapy.

Inhibition of the AI-2 and CSP systems has been proved a successful strategy to tackle *in vitro* oral biofilm formation (Wang et al., 2011; Kasper et al., 2014; Kolderman et al., 2015; Lee et al., 2015; Cho et al., 2016; Ryu et al., 2016; Muras et al., 2018, 2020b). D-galactose reduced biofilm formation in *F. nucleatum*, *P. gingivalis*, and *Tannerella forsythia* by interfering with AI-2 sensing (Ryu et al., 2016). Similarly, D-ribose and furanone, both QS inhibitors, reduced bacterial load and bone destruction in a mice infection model of *P. gingivalis* (Cho et al., 2016). When using a cell extract that interferes with AI-2-mediated QS, Muras et al. (2018) observed a significant reduction in *S. mutans* biofilm formation. Wang et al. (2011) reported that several oral streptococci, as well as oral pathogens such as *P. gingivalis*, could interfere with the CSP system in *S. mutans*, reducing biofilm formation and bacteriocin production (Wang et al., 2011). So far, these promising studies have been limited to *in vitro*

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investigations. Thus, further *in vivo* studies are needed before this approach can be considered for implementation in the clinical practice.

Most of the QSI studies are focused on the on AHL-mediated QS, specially on the enzymatic inactivation of AHLs. QQ against AHLs is commonly found among diverse bacterial taxa, including gram-positive bacteria, where it can represent a competitive mechanism against gram-negative bacteria, or a strategy of nutrient acquisition (Romero et al., 2012; LaSarre and Federle, 2013). Naturally present QQ activities in human saliva samples could point towards a role of AHL-mediated QS in oral biofilms (Muras et al., 2020b), but further confirmation of the relevance of these findings is needed.

There are three main types of enzymes able to degrade AHLs: lactonases, acylases and oxidoreductases (Figure 4). Lactonases hydrolyse the lactone ring of AHLs, rendering non-functional molecules. The hydrolysis of the lactone ring can also occur spontaneously at pH > 7, and lowering the pH can revert this effect (Yates et al., 2002). Acylases exscind the lactone ring from the acyl chain in an irreversible manner. Oxidoreductases can oxidise and reduce the acyl chain of AHLs, modifying their specificity towards AHL receptors (LaSarre and Federle, 2013).

The inhibition of AHLs to control oral biofilm formation is of special interest since numerous enzymes targeting these molecules have been reported (Mayer et al., 2015, 2018; Romero et al., 2015). Among them, Aii20J, a lactonase patented by the USC research group, has no genotoxicity (UNE-EN ISO 10993-3:2015) and no cytotoxicity in eukaryotic cells (OECD Guideline 471) (unpublished results). Besides, Aii20J is highly stable and retains its activity after 24 months in a commercial mouthwash at room temperature (unpublished results). Despite being a member of the metallo-beta-lactamase super-family, Aii20J has no effect on beta-lactam antibiotics (Mayer et al., 2015) and does not interfere with bacterial growth (Muras et al., 2018), reducing the risk of inducing antimicrobial resistance. These properties make Aii20J a promising candidate for developing antibiofilm strategies, particularly in the oral health context. The Aii20J lactonase has been tested on a six-species *in vitro* biofilm model containing *S. oralis, Veillonella parvula, A. naeslundii, F. nucleatum, A. actinomycetemcomitans,* and *P. gingivalis* (Muras et al., 2020b). The consortium responded to the addition of the lactonase enzyme by reducing biofilm coverage and viability of the cells (Muras et al., 2020b). Furthermore, the same study reported an inhibitory activity on saliva-derived microcosm biofilms upon exposure to Aii20J (Muras et al., 2020b). These microcosm

biofilms were dominated by gram-negative taxa, but studies using *in vitro* biofilms comprising a higher microbial diversity, and especially higher abundances of gram-negative anaerobes would be very interesting to assess the antibiofilm capacity of novel compounds in the context of a periodontal biofilm. As seen with QSI approaches against AI-2 and AIPs, these promising *in vitro* studies should be translated into *in vivo* and clinical trials to assess their potential.

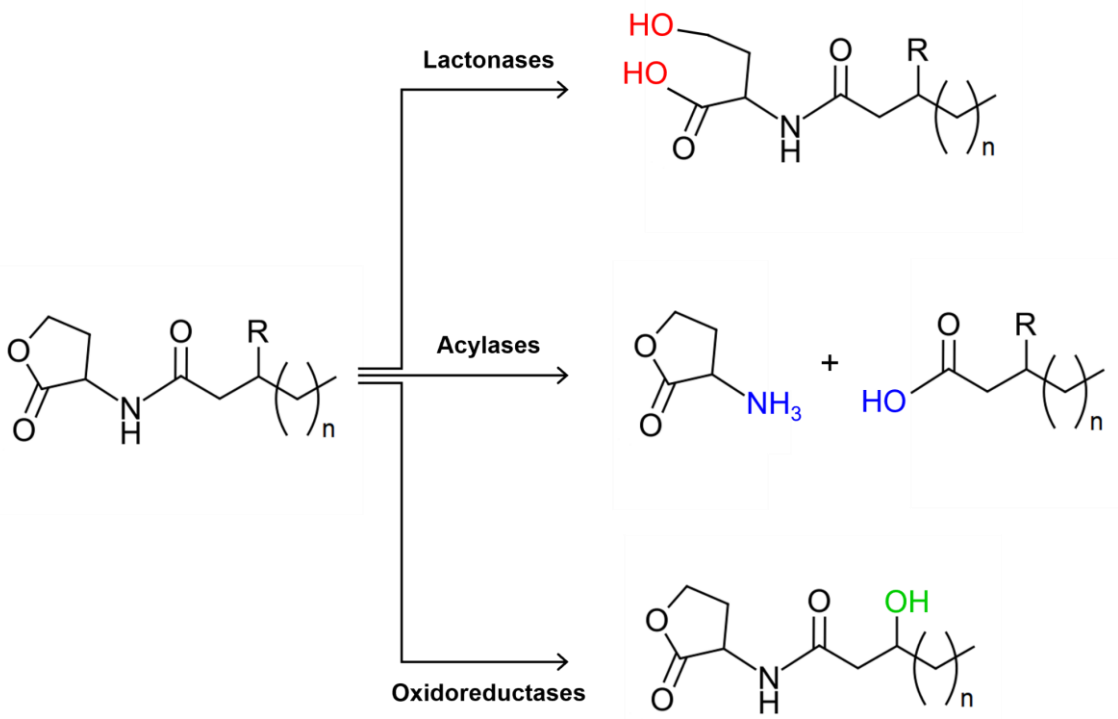


Figure 4. Action mechanism of the three main types of AHL-degrading enzymes. Modified from Utari et al. (2017).

Pathogenic bacteria are more susceptible to antibiotic therapy after treatment with QSI compounds, as assessed by different studies (Asahi et al., 2012; Simonetti et al., 2016). This finding opens the possibility of using QSI compounds as adjuvants to treat biofilm-associated infections. Before these approaches can be proposed in the clinical practice, our knowledge of microbe-microbe interactions in multispecies biofilms and the influence of QS molecules in the equilibrium between commensal and potentially pathogenic bacteria in the mouth needs to be expanded.

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4. Conclusion

The complex and multifactorial nature of caries, periodontitis, and the emerging problem of peri-implantitis, presenting no single cause, limits the efficacy of simplistic preventive solutions such as eliminating one type of organism or complete removal of the dental plaque (Philip et al., 2018). Therefore, the future of oral disease management will be based on targeted approaches to restore the microbiome equilibrium (Rosier et al., 2018) and increase the resilience of the resident homeostatic microbiota (Marsh et al., 2015; Philip et al., 2018; Rosier et al., 2018; Wade, 2021). These strategies will probably require a personalised diagnosis of the dysbiosis state of the patients.

Among the different ecological strategies that are being explored, special attention should be paid to those that seem to be already involved in maintaining the homeostasis of the healthy oral microbiota. Among these alternatives, QQ strategies appear to be promising. Nevertheless, more studies are needed to evaluate the potential of these strategies, including the establishment of reliable *in vitro* models that are cost-effective and reproducible, in which to develop these investigations. In addition, it would be interesting that these models allowed for applying several techniques to investigate different aspects of biofilm formation. These techniques could comprise imaging analysis and easy harvesting of biomass to perform nucleic acid or protein extractions for further community structure investigations, or evaluation of the differential expression of genes and proteins. These investigations are needed before translating these strategies to *in vivo* trials, which are required to confirm the potential of these novel ecological approaches aiming to maintain and restore the equilibrium of the oral microbiota.

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Objectives

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The main objective of the present PhD thesis was the investigation of QS systems on oral biofilm, focused on the potential role of AHLs in the regulation of oral biofilm formation processes. This general objective is further divided into three specific objectives:

1. To evaluate the use of the QQ enzyme Aii20J as an antibiofilm strategy against biofilms representative of the two major bacterial oral diseases: periodontitis and dental caries. This first objective comprised two sub-objectives:
    1.1. To establish a methodology of *in vitro* biofilm generation using oral samples as inocula, aiming to achieve biofilms that are an *in vitro* model representative of the initial bacterial diversity in the samples.
    1.2. To evaluate the effect of Aii20J on *in vitro* oral microcosm biofilms in terms of total biofilm mass and microbiome structure on microbially diverse biofilms containing gram-negative anaerobes and in biofilms dominated by gram-positive taxa.

2. To study AHL-mediated QS processes in oral biofilms. This second objective comprises two sub-objectives:
    2.1. To evaluate the production of AHLs in *in vitro* microcosm biofilms generated from oral samples.
    2.2. To confirm previous results on the high prevalence of QQ activity against AHLs in oral samples. Additionally, the prevalence of QS and QQ-related sequences in oral metagenomes will be examined.

3. Given the previously observed effect of the QQ enzyme Aii20J on biofilms dominated by gram-positive bacteria, we intended to characterise the effect of the exogenous addition of AHLs on a gram-positive representative, *E. faecalis*, growing in monospecies biofilms. This third objective comprises three sub-objectives:
    3.1. To characterise biofilm formation in *E. faecalis* upon exposure to AHLs.
    3.2. To evaluate the effect of AHL exposure on the transcriptional levels of genes associated with peptide-mediated QS, biofilm formation and membrane stress responses in *E. faecalis*.
    3.3. To examine the effect of AHL exposure on the proteomic profile of *E. faecalis*.

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Methods

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1. Subject recruitment and ethics statements

1.1. Recruitment of patients with periodontal disease

An initial group of seven healthy donors (female/male (F/M): 6/1, mean age: 41.1 ± 11.9 years), and ten patients with periodontal disease (F/M: 5/5, mean age: 54.1 ± 12.9 years) were recruited from the University Dental Clinic of the Faculty of Medicine and Dentistry at the University of Santiago de Compostela (ES) between December 2019 and March 2020. A second group comprising 11 healthy donors (F/M: 8/3, mean age: 51.5 ± 14.1 years), and 24 patients with periodontal disease (F/M: 11/13, 64.0 ± 13.2 years) were recruited from the University Dental Clinic of the Faculty of Medicine and Dentistry at the University of Santiago de Compostela (ES) between June 2020 and May 2022. In all cases, oral health status was assessed according to the proceedings from the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions (Papapanou et al., 2018; Tonetti et al., 2018) (Tables 3 and 4). Antibiotic treatment up to one month prior to sampling was the exclusion criterium set for this study.

The investigation protocol 2009/319 for patient recruitment and sample handling, modified in July 2017, was approved by the Ethical Committee of Clinical Investigations of Galicia (Xunta de Galicia, ES). Written informed consent was obtained from all participants.

Table 3. Classification of the oral health status of the first group of patients recruited in this study (*n* = 17). F: female. M: male. RP: reduced periodontium.

	Code	Age	Sex	Classification
Healthy (*n* = 7)	H1	30	F	Healthy periodontium
	H2	57	M	Healthy, RP
	H3	46	F	Healthy periodontium
	H4	22	F	Healthy periodontium
	H5	39	F	Healthy periodontium
	H6	49	F	Healthy, RP
	H7	45	F	Healthy, RP
Periodontal disease (*n* = 10)	P1	48	F	Periodontitis (Stage II)
	P2	49	M	Periodontitis (Stage II)
	P3	47	F	Gingivitis
	P4	43	F	Gingivitis
	P5	44	F	Gingivitis
	P6	70	M	Periodontitis (Stage IV)
	P7	75	M	Periodontitis (Stage IV)
	P8	55	F	Periodontitis (Stage II)
	P9	65	M	Periodontitis (Stage III)
	P10	39	M	Periodontitis (Stage III)

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Table 4. Classification of the oral health status of the second group of patients recruited in this study (*n* = 35). F: female. M: male. RP: reduced periodontium.

	Code	Age	Sex	Classification
Healthy (*n* = 11)	H8	24	F	Healthy periodontium
	H9	49	M	Healthy, RP
	H10	50	F	Healthy periodontium
	H11	42	F	Healthy, RP
	H12	54	M	Healthy, RP
	H13	69	F	Healthy, RP
	H14	54	M	Healthy, RP
	H15	74	F	Healthy, RP
	H16	48	F	Healthy, RP
	H17	39	F	Healthy periodontium
	H18	63	F	Healthy, RP
Periodontal disease (*n* = 24)	G1	31	F	Gingivitis
	G2	77	F	Gingivitis
	G3	72	M	Gingivitis
	P11	64	F	Periodontitis (Stage III)
	P12	78	F	Periodontitis (Stage II)I
	P13	62	M	Periodontitis (Stage III)
	P14	73	M	Periodontitis (Stage II)I
	P15	38	F	Periodontitis (Stage I)II
	P16	64	M	Periodontitis (Stage II)I
	P17	57	F	Periodontitis (Stage IV)
	P18	54	M	Periodontitis (Stage III)
	P19	70	M	Periodontitis (Stage II)I
	P20	74	M	Periodontitis (Stage II)I
	P21	64	F	Periodontitis (Stage II)I
	P22	55	F	Periodontitis (Stage I)II
	P23	65	F	Periodontitis (Stage III)
	P24	64	M	Periodontitis (Stage III)
	P25	51	F	Periodontitis (Stage III)
	P26	85	M	Periodontitis (Stage III)
	P27	70	M	Periodontitis (Stage III)
	P28	71	M	Periodontitis (Stage II)I
	P29	86	M	Periodontitis (Stage III)
	P30	51	F	Periodontitis (Stage II)I
	P31	59	M	Periodontitis (Stage II)I

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1.2. Recruitment of children with caries

A cohort of ten caries-free children (F/M: 4/6, mean age: 8.7 ± 3.2 years), and ten children with caries (F/M: 5/5, mean age: 7.8 ± 1.5 years) were recruited from a private practice in Santiago de Compostela (ES) between December 2021 and July 2022 (Table 5). Orthodontic appliances and use of antibiotics up to one month prior to sampling were the exclusion criteria set for this study. All patients recruited presented mixed dentition.

The investigation protocol 2021/495 for patient recruitment and sample handling, modified in November 2021, was approved by the Ethical Committee of Clinical Investigations of Galicia (Xunta de Galicia, ES). Written informed consent was obtained from the legal guardians of all participants.

Table 5. Classification of the oral health status of the patients recruited for the caries experiments (*n* = 20). F: female. M: male. N/A: not available.

	Code	Age	Sex	Classification
Caries-free (*n* = 10)	NCa1	3	M	Healthy
	NCa2	11	F	Healthy
	NCa3	13	M	Healthy
	NCa4	11	M	Healthy
	NCa5	N/A	M	Healthy
	NCa6	9	F	Healthy
	NCa7	8	F	Healthy
	NCa8	7	M	Healthy
	NCa9	5	F	Healthy
	NCa10	11	M	Healthy
Caries (*n* = 10)	Ca1	6	M	Caries
	Ca2	9	M	Caries
	Ca3	7	F	Caries
	Ca4	6	M	Caries
	Ca5	7	M	Caries
	Ca6	9	M	Caries
	Ca7	6	F	Caries
	Ca8	10	F	Caries
	Ca9	9	F	Caries
	Ca10	9	F	Caries

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2. Sample collection and growth conditions

2.1. Saliva and subgingival samples from healthy and periodontal disease patients

Saliva and subgingival samples were taken from seven healthy donors (codes H1 – H7) and ten patients with periodontal disease (PD) (codes P1 – P10) (Table 3). Samples were stored at 4 °C and processed promptly. Saliva samples were collected in sterile tubes and diluted 1:100 in each culture media used (Fernandez y Mostajo et al., 2017). Subgingival samples consisted of gingival crevicular fluid (GCF) collected by inserting 16 sterile paper points (4 in each mouth quadrant) in the gingival sulcus for 1 minute. In patients with PD, samples were taken from the deepest periodontal pockets. Samples were transported in 1 mL of thioglycollate broth (Merck Millipore), from which a 1:53 dilution with the corresponding medium was made (Mira et al., 2019). Samples from the initial collection (n = 17) were inoculated in 1) Brain-Heart Infusion (BHI) (PanReac) and grown in aerobic conditions, and 2) Schaedler culture medium, supplemented with vitamin K1 (0.1 mg/L) (SCH-K1) (Conda Pronadisa) and grown in anaerobic conditions. Biofilms (n = 17) were grown for 24 hours at 37 °C, refreshing culture media and treatments at 12 hours.

A broader screening of culture conditions was performed using subgingival samples, collected following the previously described methodology, from a healthy donor (H8), a patient with gingivitis (G1), and two patients with periodontitis (P11 and P12) (Table 4). Biofilm generation conditions were: 1) BHI and aerobiosis, 2) BHI and anaerobiosis, 3) SCH-K1 and anaerobiosis, and 4) semi-defined McBain medium, containing 2.5 g/L mucin, 2 g/L Bacto peptone, 2 g/L Trypticase peptone, 1 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L KCl, 0.2 g/L $CaCl_2$, 1 mg/L hemin, and 0.2 mg/L vitamin K1, with Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) at pH 7.0 (McBain et al., 2005; Janus et al., 2015) in anaerobiosis. Subsequent samples from ten healthy donors (H9 – H18), two patients with gingivitis (G2, G3), and 19 patients with periodontitis (P13 – P31) (Table 4) were grown in BHI-anaerobiosis and McBain-anaerobiosis. Biofilms (n = 35) were grown for 24 hours at 37 °C, refreshing culture media and treatments at 12 hours.

2.2. Supragingival plaque from caries-free and caries children

Supragingival plaque samples were taken from ten caries-free children (NCa1 – NCa10) and ten children with caries (codes Ca1 – Ca10) (Table 5) by scrapping with a curette the vestibular and lingual surfaces of four dental pieces. Both deciduous and permanent pieces were

sampled. Supragingival plaque was stored in 1.5 mL of thioglycolate medium (Merck Millipore) at 4 ℃ and processed promptly. Samples (n = 20) were vortexed for 30 s, and 100 µL were set aside and frozen at -80 ℃ for genomic DNA (gDNA) extraction. The remaining volume was diluted 1:53 (Mira et al., 2019) in McBain and McBain supplemented with 0.2% sucrose (McBain-sucrose). Samples were grown in the AAA model and, when necessary, Aii20 was added at 20 µg/mL. Biofilms were grown in aerobiosis at 37 ℃ for 24 hours, refreshing culture media and treatments at 12 hours.

3. Strains and culture conditions

3.1. Biosensor strains

Two *Chromobacterium* strains unable to produce AHLs but with the ability to respond to their presence were used. The AHL biosensor *Chromobacterium subtsugae* CV026, formerly *Chromobacterium violaceum* CV026 (Harrison and Soby, 2020), responds to short-chain AHLs (4 to 8 carbons) (McClean et al., 1997). *C. violaceum* VIR07 responds to long-chain AHLs (10 to 16 carbons) (Morohoshi et al., 2008). Both biosensor strains were routinely plated in Luria Bertani (LB) with 25 µg/mL of kanamycin, and grown at 30 ℃.

3.2. Oral strains

Five *E. faecalis* strains were investigated regarding their sensitivity to the addition of AHLs. The collection strain ATCC 29212 was used as a reference throughout several experiments due to the complete annotation of its genome in NCBI's genome database (National Library of Medicine (US), 2012). Additionally, the type strain ATCC 19433^{T} was also used, along with three isolates from infected dental root-canals labelled UmID4, UmID5, and UmID7. Root-canal isolates were obtained from Umeå bacterial collection (Division of Endodontics, Department of Odontology, Umeå University, SE). *E. faecalis* strains were routinely plated on BHI and grown at 37 ℃.

4. Production and purification of the QQ enzyme Aii20J

The QQ enzyme Aii20J, an AHL-lactonase enzyme obtained from the marine bacterium *Tenacibaculum* sp. strain 20J (Romero et al., 2011, 2014), was obtained as previously described (Mayer et al., 2018). Briefly, protein expression of the recombinant *E. coli* BL21(DE3)pLysS containing Aii20J was induced when suspensions reached 0.6 OD_{600nm}, by the addition of 1 mM

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Isopropyl-D-thiogalatopyranoside (IPTG) followed by further overnight incubation at 22 °C with gentle orbital shaking. The induced cells were pelleted, resuspended in phosphate buffered saline (PBS) and then lysed by sonication. Imidazole was added at a final concentration of 20 mM to prevent non-specific binding during later purification steps. Purification of Aii20J was achieved using the His GraviTrap affinity column protein purification kit (GE Healthcare) following manufacturer's instructions. Briefly, the columns have a nickel-based medium for the purification of histidine-tagged proteins by immobilized metal affinity chromatography. The first step was the equilibration of the column with a phosphate buffer (10 mM $Na_2HPO_4 \cdot 2 H_2O$, 10 mM $Na_2H_2PO_4 \cdot H_2O$, 500 mM NaCl) containing imidazole in the same concentration as in the sample (20 mM) to prevent binding of host cell proteins with exposed histidines to the nickel-based medium. After sample flow-through, a washing step was performed with the 20 mM-imidazole buffer. Last, elution of the affinity-bound protein was achieved with a 500 mM-imidazole buffer. Remaining imidazole was then removed from the eluted sample by dialysis with D-tubes (MWCO 10 kDa) (Merck Millipore) in sterile Milli-Q water.

When appropriate, the QQ enzyme Aii20J was added to the assays to a working titre of 20 µg/mL, corresponding to 10 times the minimum concentration of purified enzyme needed to degrade 10 µM of a given AHL in 3 hours. AHL-degradation capacity of Aii20J, as well as remaining Aii20J activity in biofilm supernatants, was routinely verified using a chromogenic assay (see section 7.2). Additional verifications of Aii20J activity against AHLs were done with high-performance liquid chromatography–mass spectrometry (HPLC-MS) analyses, performed by the team at the Unit of Chromatographic Techniques and Water Analysis (University of A Coruña, ES).

5. Preparation of QQ acylase enzymes

Acylase I from porcine kidney (Merck) and acylase I from *Aspergillus melleus* (Merck) were dissolved in PBS, filtered through 0.22 µm filters (Sartorius Stedim Biotech GmbH, Goettingen, DE) and stored at 4 °C. Acylase I from porcine kidney has a lower activity as compared with acylase I from *Aspergillus*, as specified by the manufacturer (Merck). Thus, when added to biofilm assays, acylase I from porcine kidney was used at a working titre of 2 U/mL, while acylase I from *Aspergillus* was used at 0.02 U/mL. Acylase I from *Aspergillus* was used together with 10^{-3} mM of $CoCl_2$ (Merck, Sigma-Aldrich), as this cofactor is required

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for its enzymatic activity (Merck, Sigma-Aldrich). For this purpose, a solution of $CoCl_2$ was prepared in Milli-Q water, filtered through 0.22 μm filters and stored at 4 °C.

6. Preparation of acyl-homoserine lactones (AHLs)

The AHLs used were: *N*-Butyryl-DL-homoserine lactone (C_4-HSL; Fluka, Thermo Fisher Scientific, Waltham, US), *N*-(3-Oxobutyryl)-L-homoserine lactone (OC_4-HSL; University of Nottingham, Nottingham, GB), *N*-Hexanoyl-L-homoserine lactone (C_6-HSL; Sigma-Aldrich, Merck KGaA, Darmstadt, DE), *N*-Oxohexanoyl-L-homoserine lactone (OC_6-HSL; University of Nottingham), *N*-Octanoyl-L-homoserine lactone (C_8-HSL; Sigma-Aldrich), *N*-Decanoyl-L-homoserine lactone (C_{10}-HSL; Sigma-Aldrich), *N*-Dodecanoyl-L-homoserine lactone (C_{12}-HSL; Sigma-Aldrich), *N*-Tetradecanoyl-DL-homoserine lactone (C_{14}-HSL; Fluka), *N*-Hexadecanoyl-L-homoserine lactone (C_{16}-HSL; University of Nottingham), and *N*-Octadecanoyl-L-homoserine lactone (C_{18}-HSL; University of Nottingham).

Stock solutions for each AHL were prepared in acetonitrile. AHLs were dissolved to a concentration of 1 mg/mL and stored at -20 °C, only C_{16}-HSL and C_{18}-HSL had to be dissolved to 0.1 mg/mL due to their lower solubility. AHLs were either used individually at a working concentration of 1 μM, or clustered in two groups according to their acyl chain length, i.e., either short-chain AHLs (bearing between 4 and 8 carbons; C_4-HSL, OC_4-HSL, C_6-HSL, OC_6-HSL, and C_8-HSL) or long-chain AHLs (bearing between 10 and 18 carbons; C_{10}-HSL, C_{12}-HSL, C_{14}-HSL, and C_{18}-HSL). Prior to each experiment, intermediate dilutions of these stock solutions were prepared in sterile water to generate mixes of the respective short- and long-chains AHLs, reaching a total concentration of 20 μM for each AHL. AHL mixes were stored at -20 °C.

7. Quorum Quenching activity solid plate assays

Solid plate bioassays were used throughout several experiments to assess: the presence of QQ activity in saliva samples from healthy donors and patients with PD, and the AHL-degradation activity of the QQ enzyme Ai20J, both purified and in biofilm supernatants after the incubation time.

7.1. Quorum Quenching activity in saliva samples

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The presence of QQ activity in saliva samples was tested using solid plate assays carried out with the AHL biosensors *C. subtsugae* CV026 for C_6-HSL (McClean et al., 1997), and *C.*

violaceum VIR07 for C_{12}-HSL (Morohoshi et al., 2008). Saliva samples were split into two fresh 1.5 mL vials and mixed with C_6-HSL (10 µM) and C_{12}-HSL (10 µM). The pH of the saliva samples was checked beforehand and acidified when it exceeded pH 7 to prevent spontaneous opening of the lactone ring of the AHLs (Yates et al., 2002). PBS pH 6.5 with the corresponding AHL (10 µM) was used as negative control (Muras et al., 2018). All mixtures were incubated for 24 hours at 22 °C for the saliva samples to be in contact with the AHLs.

The presence of functional, i.e.; non-degraded, AHLs was detected by adding a mixture of an overnight culture of the corresponding biosensor and soft LB (0.8% agar) in a 1:4 proportion on top of an LB agar plate. Wells were manufactured onto the agar, and 100 µL of sample were placed in each well. Plates were incubated for 24 hours at 30 °C. The lack of violet pigmentation, as opposed to the violet halo observed around the negative controls, indicated AHL degradation.

7.2. Quorum Quenching activity of the QQ enzyme Aii20J

To evaluate the AHL-degradation activity of the QQ enzyme Aii20J, ten-fold serial dilutions of the purified enzyme were made, until reaching a titre of 0.02 µg/mL of enzyme. The established minimum active concentration (MAC) of Aii20J is 2 µg/mL, and it was routinely tested to check for the reproducibility of these values among batches of enzyme. For biofilm supernatants containing the enzyme Aii20J at a titre of 20 µg/mL, 100 µL were transferred to a fresh microtube at the end of the incubation time. Both the purified Aii20J and biofilm supernatants containing Aii20J were incubated with 10 µM of C_6-HSL for 3 hours at 22 °C, and solid plate assays were carried out as seen in the previous section 7.1.

carried out as seen in the previous section 7.1.

8. Minimum Inhibitory Concentration solid plate assay

Overnight *E. faecalis* ATCC 29212 suspensions were plated on BHI. Sterile paper discs bearing AHLs at 100 µM (100-fold higher than the concentration used in biofilm assays) were placed on top of the agars. Discs were dried under sterile laminar flux to eliminate the acetonitrile in which AHLs were dissolved, prior to the solid plate assay. Plates were incubated at 37 °C for 24 hours before the appearance of inhibition halos surrounding the discs was assessed. Homogenous growth surrounding the disc indicated no inhibitory activity of the AHL.

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9. Biofilm generation and quantification

9.1. Generation and quantification of microcosm biofilms from oral samples

9.1.1. Biofilms originated from saliva and subgingival samples

In vitro biofilms originated from saliva and subgingival samples were generated and measured using two different methodologies. The first is a modification of the Amsterdam Active Attachment model (AAA model) (Exterkate et al., 2010; Muras et al., 2020a). Briefly, the lids of 12-well cell culture plates (VWR) were replaced by custom-made stainless-steel lids onto which 12 silicone tubes were attached allowing for the insertion of glass coverslips (18 × 18 mm) (Menzel Gläser, Braunschweig, DE) to serve as substrata for biofilm formation (Muras et al., 2020a). The glass coverslips fit vertically into the wells of the culture plates, filled with 3 mL of the corresponding inocula, meaning half of the coverslip surface is submerged. The vertical disposition of the coverslips prompt the active attachment of bacterial cells onto their surface. The AAA model allows the refreshment of culture media and treatments by transferring the lid with the glass coverslips to a new plate. When required, an anaerobic atmosphere was achieved using an anaerobiosis jar (OXOID, Basingstoke, GB) with AnaeroGen 2.5L Atmosphere Generation System sachets (OXOID). Biofilms were quantified by crystal violet (CV) assay. After incubation, coverslips were allowed to dry in a fresh plate, and biofilms were stained for 20 minutes with 2 mL of a 0.04% CV solution (v/v in distilled water) (Panreac, Barcelona, ES). The excess dye was removed by washing twice with distilled water. Before releasing the CV for quantification, pictures were taken to perform visual examinations of the biofilms. When two independent observers found control and Aii20J-treated biofilms to be visually distinguishable, the biofilms were considered to have macroscopical differences (Figure 5). Finally, CV bound to the biofilms was diluted in 3 mL of a 33% acetic acid solution (v/v in distilled water) (Scharlab, Barcelona, ES). Sample absorbance was measured at 590 nm. All experiments were performed in triplicate.

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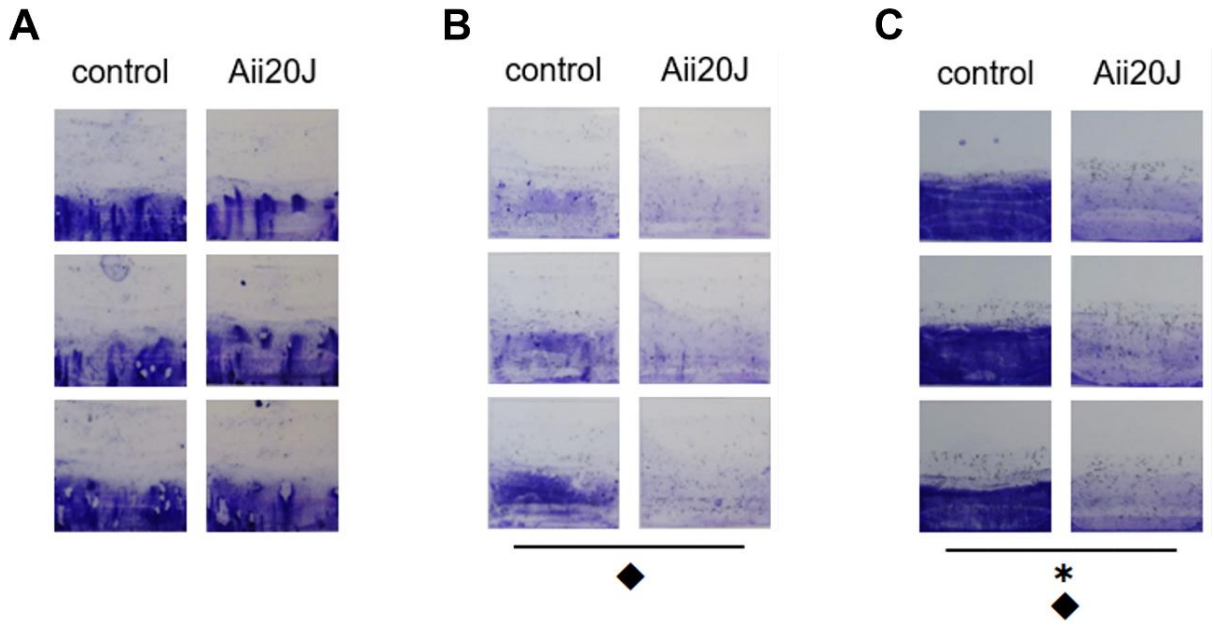


Figure 5. Visual examination of biofilms grown in the AAA model and stained with CV. A. Control and Aii20J-treated biofilms that do not differ statistically nor visually. B. Control biofilms that are not significantly different in absorbance (OD_{590nm}) but differ visually (marked with a diamond; ♦) from Aii20J-treated biofilms. C. Control biofilms that differ statistically (*) and visually (♦) from Aii20J-treated biofilms. Reproduced with permission from Parga et al. (submitted).

When necessary, the Aii20J lactonase was added to the biofilm assays at a final concentration of 20 µg/mL. Negative controls received sterile Milli-Q water. Two additional controls were performed: to obtain the first one, the purified Aii20J (35 kDa in size) was filtered through a Centricon with a molecular weight cut-off of 10 kDa (Merck) to remove the enzyme, recovering the remaining soluble fraction. The other additional control was a heat-inactivated Aii20J (90 min at 121 °C) that did not conserve AHL-degrading activity (see Figure 14, in Chapter 1).

Biofilms from the first group of patients (n = 17) were also generated and analysed using the xCELLigence Real Time Cell Analyzer (RTCA) System (ACEA, Biosciences Inc., San Diego, US) (Muras et al., 2018, 2020b; Mira et al., 2019). The xCELLigence measures changes in impedance as cells attach to the bottom of E-plates (ACEA, Biosciences Inc.), and the software computes cell index (CI) values that correspond to the amount of biofilm formed. E-plates 16 were inoculated with 100 µL of the corresponding culture medium and 80 µL of the saliva/subgingival inocula. To lactonase-treated samples, 20 µL of Aii20J (to a final concentration of 20 µg/mL) were added, while 20 µL of sterile distilled water were added to

the control wells. An overlay of sterile paraffin was added when an anaerobic atmosphere was required. The system was incubated at 37 °C for a total of 24 hours, with impedance measurements performed every 15 minutes. All experiments were done in triplicate.

9.1.2. Biofilms originated from supragingival plaque samples

Supragingival plaque samples were inoculated in the AAA model (Exterkate et al., 2010; Muras et al., 2020a), assembled with glass coverslips. After incubation, biofilms formed onto the glass coverslips were CV-stained and quantified as described in section 9.1.1. When necessary, Aii20J lactonase was added to the biofilm assays at a final concentration of 20 µg/mL. Negative controls received sterile Milli-Q water.

9.2. Generation and quantification of monospecies E. faecalis biofilms

The investigations on the effect of AHL exposure on *E. faecalis* biofilm formation have been done in collaboration with Dr D. Manoil, under the supervision Prof. G.N. Belibasakis in the Department of Dental Medicine, at Karolinska Institutet (SE).

Biofilms were generated in two different systems: in the AAA model (Exterkate et al., 2010; Muras et al., 2020a), and in 24-well cell culture plates (Sarstedt). In both systems, bacteria retrieved from agar plates were inoculated into 8 mL BHI (OXOID) and incubated over-night at 37 °C. Over-night *E. faecalis* suspensions were pelleted by centrifugation at 4'200 rpm for 5 min. The culture medium was removed, and the pellet resuspended into fresh BHI to spectrophotometrically adjust optical density (OD_{600nm}) at 0.5 (approx. 10^8 CFU/mL) (UV-1800 spectrophotometer, Shimadzu, Kyoto, JP).

For biofilms generated in the AAA model, 12-well plates (Sarstedt AG & Co. KG, Nümbrecht, DE) were used. Biofilms were generated by pouring 2.7 mL of fresh BHI broth in the wells, and then adding 300 µL of the 0.5 OD *E. faecalis* suspension. Biofilms were generated onto glass coverslips inserted in the lid of the AAA model, and incubated at 37 °C. Culture medium was renewed every 12 hours. Biofilms were quantified at 6, 12, 48, and 72 hours of incubation time. Biofilm staining and quantification by CV assay was performed as explained in section 9.1.1.

Biofilms were also generated onto the bottom of 24-well polystyrene plates. For that purpose, all wells were filled with 1.8 mL BHI broth, and 200 µL of the adjusted *E. faecalis* suspension were added to each well. Plates were incubated at 37 °C in an aerobic atmosphere. Biofilm formation was assessed at 2, 4, 6, 12, and 24 hours of incubation. Following incubation,

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supernatants were removed by aspiration and biofilms were washed three times with PBS. All wells were then stained with 2 mL of a solution of 0.04% crystal violet (CV) (Sigma-Aldrich) at RT. After 20 min of staining, the excess of dye was removed by aspiration and biofilms were again washed three times with PBS. The stained biofilm was detached by adding 2 mL of 33% acetic acid, and the CV incorporated in the biomass was measured spectrophotometrically at 595 nm.

When necessary, AHLs were added to the suspensions. Short- or long-chain AHL mixes were added to a working concentration of 1 μM for each AHL (acetonitrile fraction between 0.1 – 0.5%). Individual AHLs were added to a working concentration of 1 μM for each AHL (acetonitrile fraction between 0.02 – 0.22%). Corresponding controls received sterile water with the same final fractions of acetonitrile. For the assessment of the impact of AHL exposure, plates were incubated for 6 hours at 37 °C. Biofilm processing prior to CV staining was performed in the same fashion as previously described.

10. Assessment of AHLs addition on planktonic growth

To evaluate whether AHLs may impact planktonic growth, *E. faecalis* proliferation was monitored over time in 96-well plates (BRAND GMBH + Co. KG, Wertheim, DE) in the presence of AHLs. The effect of either AHL mixes or individual AHLs was evaluated. For AHL mixes, 170 μL of fresh BHI were inoculated with 20 μL of an overnight *E. faecalis* suspension adjusted at OD_{600nm} 0.5. Ten microliters of either short- or long-chain AHL mixes were then added to reach a final working concentration of 1 μM for each AHLs, as for biofilms experiments. For individual AHLs, 180 μL of fresh BHI were inoculated with 20 μL of an *E. faecalis* suspension at OD_{600nm} 0.5. Two microliters of the corresponding AHLs (3.3 μL in the case of C_{18}-HSL) were then added to reach a final working concentration of 1 μM for each AHL. For both experiments, controls received sterile water with the same final fraction of acetonitrile as in the AHL condition. Incubation was performed in a heated microplate reader (MultiSkan SkyHigh, Thermo Fisher Scientific), with orbital shaking at 37 °C. Readings at OD_{600nm} every 10 minutes were used to monitor bacterial growth.

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11. Investigation of the microbial composition of biofilms from oral samples

11.1. Subgingival in vitro biofilms from healthy and periodontal disease patients

11.1.1. Harvesting of biofilms and DNA extraction

Genomic DNA for sequencing analysis was obtained from biofilms grown in the AAA model. Biofilms were harvested by transferring the glass coverslips into 5 mL of sterile PBS. Then, they were sonicated for 15 minutes to disperse the biomass. Genomic DNA extraction was done using the "DNeasy PowerBiofilm Kit" (Qiagen), following the manufacturer's instructions. Briefly, biofilms were subjected to chemical and mechanical lysis. Proteins and inhibitors were removed, followed by pH-driven precipitation of large insoluble macromolecules. Total gDNA was captured using a silica spin filter column and finally eluted. DNA concentration was measured using a NanoDrop (Thermo Scientific).

11.1.2. Library preparation and microbiome analysis

The library preparation and sequencing were performed in the sequencing service of the Foundation for the Promotion of Health and Biomedical Research of Valencia Region (FISABIO) (ES). Microbial gDNA (5 ng/µL in 10 mM Tris pH 8.5) was used to amplify the 16S rRNA gene V3-V4 hypervariable regions. After size verification, the libraries were prepared according to the supplier's protocol (Illumina, Inc.), and the resulting 11 pM 20% on PhiX pooled library was sequenced using a 2x300 base pairs paired-end run (MiSeq Reagent kit V3(MS-102-3001)) on a MiSeq Sequencer. Quality assessment was performed using the prinseq-lite program (Schmieder and Edwards, 2011). The pipeline DADA2 (Callahan et al., 2016) was used to analyse and cluster the sequences into amplicon sequence variants, classified to the genus level with the SILVA database (Quast et al., 2013). Computations and statistics were carried out in R RStatistics (R Core Team, 2022) using knitr, knitcitations, markdown (Allaire et al., 2014; Boettiger, 2014; Xie, 2014), biostrings, and vegan (Oksanen et al., 2017).

Relative abundance analyses and diversity index analyses were performed in collaboration with Dentaid Research Center (ES). The differential relative abundance of the bacterial genera identified was assessed with the packages Phyloseq and DESeq2 (Love et al., 2014). Differences were filtered with a log2 fold-change threshold higher than 2 and by their base mean value, discarding the lower quartile. Beta diversity was studied using a Principal Coordinate Analysis (PCoA) of weighted UniFrac distances using the package Phyloseq.

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Differences between samples, grouped according to the studied variables, were analysed using the PERMANOVA test implemented in the adonis function of the package vegan (Oksanen et al., 2017). Alpha diversity was analysed with the R package Phyloseq (McMurdie and Holmes, 2013), using the Chao1 and the Shannon and Simpson indexes as richness and diversity estimators. The data's normality and homoscedasticity were assessed before choosing parametric or nonparametric tests included in the package stat (R Core Team, 2022).

11.1.3. Bacterial quantification by qPCR

The quantification of periodontopathogens by qPCR was performed in collaboration with Dentaid Research Center (ES). The amount of total bacteria, *P. gingivalis* and *P. endodontalis* present in subgingival periodontitis-derived biofilms grown in McBain-anaerobiosis was evaluated by qPCR in a LightCycler 480 II Thermocycler (Roche Diagnostics GmbH). The reactions were performed in a 20 µL volume containing LightCycler 480 II Probes Master (Roche Diagnostics GmbH), specific primers and probes for each bacteria or universal primers for Eubacteria (Table 6), 5 µL of isolated DNA and PCR grade sterile water. Positive and negative controls and sample reactions were performed in duplicate.

Table 6. Sequences of the sets of primers (F: forward; R: reverse) and probes (P) used for the quantification of each bacterial species. The last columns indicate the normalised concentrations and the amplicon size. Modified from Parga et al. (submitted).

Species	Target gene	Sequence	Concn (µM)	Length (bp)
P. endodontalis	rpoB	F: TGTTAAGGATGGGAAAGTCGAT R: GCGTAGCGTGACTTTACTTTGTC P: #6[a]	0.8 0.8 0.2	147
P. gingivalis[b]	rgpB	F: GCCTTACCGTCTTTCACGAT R: AAGATGCTCGACACATGGAC P: CCATCGTCAGAGAGCGTAGC[c]	0.5 0.5 0.2	206
Eubacteria[d]	16S rRNA	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGGTATCTAATCCTGTT P: CGTATTACCGCGGCTGCTGGCAC	0.6 0.7 0.2	462

[a] Universal ProbeLibrary probe (Roche).
[b] Described at Vilarrasa et al. (2018).
[c] TAMRA TaqMan probe (Life Technologies) labeled with 6FAM.
[d] Described at Nadkarni et al. (2002). All other primers and probes were designed by the team at Dentaid Research Center.

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The samples underwent an initial amplification cycle of 95 °C – 10 min, followed by 40 cycles of 95 °C – 10 s, annealing at 60 °C – 30 s and extension at 72 °C – 1 s. Crossing points were calculated by LightCycler 480 Software 1.5 (Roche Diagnostics GmbH) using the second derivative maximum method. Crossing point values were then extrapolated to colony-forming units using standard curves, previously established from 10-fold serial dilutions of DNA extracted from pure bacterial cultures in log phase.

11.2. Supragingival plaque in vitro biofilms from caries-free and caries patients

11.2.1. Harvesting of biofilms and DNA extraction

Harvesting of the *in vitro* biofilms for gDNA extraction was done following the methodology described in section 11.1.1.

11.2.2. Library preparation and microbiome analysis

The library preparation and Illumina sequencing were performed in the sequencing service of FISABIO (ES), as described in section 11.1.2.

Relative and differential abundance analyses were performed in collaboration with Dr Balboa from the Center of Cross-disciplinary Research in Environmental Technologies (CRETUS) (University of Santiago de Compostela, ES). The Linear Discriminant Analysis (LDA) of the bacterial genera identified was assessed with the packages Phyloseq and DESeq2 (Love et al., 2014). Only differences with a log fold-change either below 2 or above 2 were considered relevant.

12. Effect of the addition of AHLs on regulation of gene transcripts in *E. faecalis*

The evaluation of the transcriptional levels of a selection of *E. faecalis* genes has been done in collaboration with Dr D. Manoil, under the supervision Prof. G.N. Belibasakis in the Department of Dental Medicine, at Karolinska Institutet (SE).

12.1. RNA extraction and generation of a complementary DNA library

E. faecalis biofilms were grown and exposed to either short- or long-chain AHL mixes, as previously described in section 9.2. After 6 hours of incubation, the biofilms were washed three times with BHI (pre-heated at 37 °C) and immediately frozen at -80 °C. RNA extractions were performed using the RiboPure Bacteria RNA Purification Kit (Thermo Fischer Scientific),

following the manufacturer's instructions. In brief, a solution of phenols and chaotropic salts was directly poured onto the frozen biofilms to already protect them from RNase activity while defrosting. Biofilms were then detached by vigorously pipetting the solution in and out. The entire content of each well (detached biofilms in the phenol solution) was transferred into zirconia beads-containing tubes, and bacteria were lysed by bead-beating. Chloroform was then added to separate the organic and aqueous phases. The aqueous phase was transferred to a clean tube to precipitate the RNA fraction with 99.9% molecular grade ethanol. Further purification steps were performed using the provided glass-fibre columns, and RNA was finally eluted by passing twice 30 µL of the elution solution, preheated at 65 °C. A DNase I treatment was ultimately performed to eliminate potential gDNA carryover. RNA yields were measured using a NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific), and stored at -80 °C.

Reverse-transcription (RT) was performed using the SuperScript IV VILO Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, reactions were performed in 20 µL total volume that comprised 4 µL of the SuperScript IV VILO Master Mix, up to 16 µL of template RNA and completing up to the final volume with nuclease-free water when necessary (Thermo Fisher Scientific). The volume of input RNA templates was optimised for each strain to normalise the RNA quantity used in RTs, i.e., 352 ng for ATCC 29212, 24 ng for ATCC 19433T, 132 ng for UmID4, 160 ng for UmID5 and 320 ng for UmID7. Reverse-transcription was performed in a cycle comprising an annealing step at 25 °C for 10 min, an RT step at 50 °C for 10 min, and a final enzyme inactivation step at 85 °C for 5 min. Non-RT controls were also run to ensure the absence of gDNA carryover. The generated complementary DNA (cDNA) was titrated fluorometrically by Qubit 3.2 (Thermo Fisher Scientific) using the dsDNA HS assay kit (Thermo Fisher Scientific), and stored at -20 °C.

12.2. Primer design

Primers were designed using the NCBI primer design tool by aligning sequences onto the reference genome of *E. faecalis* ATCC 29212 (GenBank accession number: GCA_000742975.1). Additional BLAST alignments against the reference genomes of strains ATCC 19433^{T} (GCA_000392875.1), V583 (GCA_000007785.1), and 39EA1 (GCA_003319815.1), were performed to bioinformatically verify that primers anneal to the target loci. Furthermore, preliminary qPCRs were run to validate that each primer pair yielded

a single amplicon product, which melting temperature (T_m) corresponded to the predicted one. Table 7 displays the characteristics of each primer pair. Target genes were didactically clustered into three categories based on their function, i.e., QS- and virulence-related genes, biofilm-related genes, and membrane stress responses.

12.3. qPCR workflow and relative quantification of target transcripts

Transcriptional levels of the targeted transcripts were assessed by qPCR. Reactions were performed in 20 µL total volume that comprised 10 µL of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), 6 µL of nuclease-free water, 1 µL of each forward and reverse primer (0.2 µM working concentration) and 2 µL of cDNA (corresponding to a 2.5% working dilution). qPCR constituents were mixed in MicroAmp Optical 96-Well Reaction Plates (Thermo Fisher Scientific) and reactions were run in an Applied BioSystems 7500 Fast Real-Time PCR thermocycler (Thermo Fisher Scientific). The amplification protocol consisted in two consecutive holding stages, at 50 °C for 20 s, and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Melting curves were run at the end of each experiment with the following settings: 95 °C for 15 s, 60 °C for 1 min, a temperature transition slope of 1%, 95 °C for 30 s, and 60 °C for 15 s.

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Table 7. Primer pairs used in this study. The table includes the target genes, forward (F) and reverse (R) primer sequences, the length of their amplicon product, the melting temperature (T_m) and the gene annotation on the reference genome of *E. faecalis* ATCC 29212 (GenBank assembly accession: GCA_000742975.1), accessed on NCBI (date: 28.09.2022).

Gene	Forward primer	Reverse primer	Amplicon size (bp)	T_m (°C) (F/R)	Gene annotation	Reference
ace	CGGATCGACAAGGAAGTGGT	CCTTGTTGCTCAAACTCGGC	109	59.75 60.04	DR75_173	(Manoil et al., 2022)
ebpA	CGTTTCAGCCATTAGCCACG	CTTCACGCCAGGTGCTTTTC	74	59.90 60.04	DR75_165	This study
efaA	CCGTTACCAGAAGACATTGCG	TAAACCAGCCATTTCCGCCT	91	59.61 59.96	fimA	This study
epaQ	GCGATGTCTTTGGACACGAC	AATGAAGAGCGCCCGATAGC	81	59.56 60.60	DR75_1013	This study
fsrC	GCCAACAAACGAATCACAACC	ACCGCAAAGCAAGCAAAACT	98	58.88 59.82	DR75_811	(Manoil et al., 2022)
σ^V	AGCTTCTCGTTTCTTTTTACGCC	TGAAAAGGCACTGAAGGCCA	118	60.06 60.11	DR75_1896	(Manoil et al., 2022)
groEL	GCACCGACTTTAACGACAGC	ACCAAATCGGCGAAACAACG	97	59.84 60.04	groL	This study
asa1	ACGCAATCCACAAAGTGCTG	CCTGCTTGCTGACTATCGCT	84	59.69 60.18	DR75_2177	This study
cylM	TGGTGACAGGGCTAGTACCAT	ACGCACTAAGGTTAACCCCTT	78	60.27 59.30	lanM *	This study
cylA	ACACGATTGCTCCAAGAGTGA	CCATCTGTCCCATCCATCACC	58	59.66 60.20	DR75_2957 *	This study
16S rRNA	CCGAGTGCTTGCACTCAATTGG	CTCTTATGCCATGCGGCATAA	137	62.58 58.85	DR75_111 (rRNA-16S)	(Manoil et al., 2022)

* Sequence annotation in the Plasmid 1 of *E. faecalis* ATCC 29212 (GenBank sequence accession: CP008815.1).

13. Proteomic analysis

The analysis of *E. faecalis* proteomic profiles was done in collaboration with Dr A. Trueba from the research group of Prof. J. Lema, in the Center of Cross-disciplinary Research in Environmental Technologies (CRETUS), at the University of Santiago de Compostela (ES).

13.1. Protein extraction

13.1.1. Biofilm harvesting

Monospecific biofilms were generated onto the bottom of 24-well plates (i.e.: 1.82 cm^2 of growth area) for 6 hours, following the methodology described in section 9.2. Biofilms were washed three times with 1 mL of BHI (pre-heated to 37 °C) and immediately frozen at -80 °C. Biofilms were harvested by adding 350 µL of extraction buffer (50 mM Tris buffer, 1% SDS, pH 7.5) to the biofilms, scratching the bottom of the wells and pooling 12 technical replicates together. Samples were kept at -80 °C or in ice before further processing.

13.1.2. Protein extraction and purification

Samples were incubated at 90 °C for 20 minutes, with shaking at 1000 rpm. After this, cell lysis was achieved by mechanical beating of the samples in tubes containing glass beads and placed in a cell disruptor (Thermo Fisher Scientific). Three cycles of 4 minutes-bead beating alternated with 1 min incubations in ice were performed. Samples were then centrifuged for 20 minutes at 3270 g at 4 °C, and the supernatants were transferred to a fresh tube for protein precipitation.

To remove organic contaminants, as well as salts and buffer residues, two steps of acetone-precipitation were performed. For each precipitation step, 4 volumes of -20 °C-cold acetone were added to each volume of protein sample. Following, incubation of the samples at -20 °C freezer for 30 minutes and centrifugation for 10 minutes at 10’000 rpm. After, acetone was carefully removed and pellets were allowed to dry at RT before repeating the precipitation step. Dry, pelleted proteins were stored at -20 °C.

13.1.3. Protein quantification

Ultrapure water (Thermo Fisher Scientific) was added to resuspend the protein pellets before protein quantification with the BCA Protein Assay Kit (Thermo Fisher Scientific).

Briefly, 5 µL of each sample were mixed with 100 µL of working reagent (reagents A and B in a 50:1 proportion) and incubated for 30 minutes at 37 ºC. Blanks and standards for the quantification curve were also prepared and processed in parallel. Samples were cooled to RT and then measured in the Nanodrop (Thermo Fisher Scientific) in BCA Assay (540 nm) mode, 6 times each. Protein concentration was calculated using the standard curve generated.

13.1.4. SDS-PAGE electrophoresis

To assess the similarity in between biological replicates and the quality and integrity of the samples, protein electrophoresis in denaturing conditions was performed. Samples were prepared following the recipe displayed in Table 8, calculating protein sample volumes to load 7 µg of protein per gel lane. Before loading the samples, they were incubated at 70 ºC for 10 min.

Table 8. Volumes required for the preparation of the samples prior to SDS-PAGE electrophoresis. "X" is the sample volume that ensures the loading of 7 µg of protein per gel lane.

Component	Volume
NuPAGE LDS Sample Buffer (4X)	(2.5 · **X**) µL
NuPAGE Reducing Agent (10X)	1 µL
Protein sample	**X** µL (7 µg of protein)
Deionized water	(6.5 - **X**) µL
Total volume	10 µL

For the electrophoresis, NuPAGE Bis-Tris 4 – 12% gels (Thermo Fisher Scientific) were used with 1X Running Buffer (20X Running Buffer was prepared with 50 mM MES, 50 mM Tris Base, 0.1% SDS and 1 mM EDTA, pH 7.3). After sample incubation, 10 µL of sample were loaded in each well. Three microlitres of pre-stained protein marker were loaded on each side of the gel. The electrophoresis was run at 200 V for 30 min. After completion of the electrophoresis run, the gel was washed with distilled water prior to staining.

13.1.5. Blue Coomassie R-250 staining protocol

After electrophoresis, gels were incubated with prefixation solution (40% vol/vol distilled water, 50% vol/vol methanol, 10% vol/vol acetic acid) for 30 minutes in a rocking

shaker. After that, gels were washed with distilled water and incubated in 0.1% Coomassie R-250 staining solution (65% vol/vol distilled water, 30% vol/vol Coomassie R-250 dissolved in methanol, 5% vol/vol acetic acid) for 30 min. Following the staining step, gels were washed again and incubated with destaining solution (82.5% vol/vol distilled water, 10% vol/vol ethanol, 7.5% vol/vol acetic acid). Destaining solution was renewed each hour until the background of the gels appeared clear.

13.2. Shotgun proteomic analysis by mass spectrometry

Proteomic analyses were performed at the Mass Spectrometry and Proteomics facilities of the University of Santiago de Compostela (ES). The protein samples were processed in solution by enzymatic digestion with trypsin and further desalination using ZipPlates C18 (Merck Millipore). The obtained sample was analysed with the nano-UHPLC-Tims-QTOF technique. Peptide samples (100 ng of protein) were injected onto a timsTOF Pro (Bruker, Bremen, DE) equipped with a nano-electrospray source (CaptiveSpray) and a tims-QTOF analyser.

MS/MS spectra were processed with PEAKS Studio Xpro (Bioinformatics Solutions, Waterloo, ON) software for protein identification. The database used contained the sequences available in NCBI's protein database for *E. faecalis* ATCC 29212 taxonomy (NCBI Taxonomy ID: 1201292). The Label-Free module from PEAKS Studio Xpro was used for protein quantification.

13.3. Protein relative abundance analyses

Proteins identified in the samples were filtered prior to differential abundance analyses. Inclusion criteria were the identification of more than one unique peptide for each protein, and the identification of each protein in at least two of the three biological replicates assessed. The spectral count (SC), i.e., the total number of spectra identified for each protein, were used to calculate fold-change values of protein abundance, as $(SC_{(C18\text{-}HSL)} - SC_{(Control)}) / SC_{(C18\text{-}HSL)}$. Fold-change values either lower than -0.5 or higher than 0.5 were considered to be biologically relevant differences in protein abundance.

Proteins that appeared only in one of the conditions were analysed by retrieving the peptides identified for each protein. NCBI's identifier codes for each peptide were translated into UniProt codes using the UniProt IDMapping tool (https://www.uniprot.org/id-mapping) (Huang et al., 2011a). Uniprot codes corresponding to the proteins' peptides were investigated

for functional annotations in the Uniprot database (https://www.uniprot.org/) (The UniProt Consortium, 2021) and using the DAVID software (https://david.ncifcrf.gov/home.jsp) (Sherman et al., 2022).

14. AHL extraction and preparation for HPLC-MS analysis

To detect the presence of AHLs at low concentrations in samples, polar molecules were extracted and concentrated to be analysed by HPLC-MS. Samples were transferred to a clean tube (VWR , US) and acidified with HCl 1 M to induce the circularisation of the lactone rings (Yates et al., 2002). To perform the organic extraction, all materials used were either high-performance polypropylene tubes (VWR), or glass tubes (Pyrex). Samples were extracted sequentially with 2 volumes of ethyl acetate (Scharlab, Barcelona, ES) and 2 volumes of dichloromethane (Scharlab) (Muras et al., 2020a). The organic phases were evaporated in a rotary evaporator (Büchi), and the dry samples were resuspended in 1 mL of acetonitrile (Merck) and stored at -20 °C until its quantification by HPLC-MS. Fresh culture media were also extracted following the same procedures.

HPLC-MS analyses were performed at the Unit of Chromatographic Techniques and Water Analysis (University of A Coruña, ES), using an HPLC 1100 series (Agilent). The mass spectrometer (MS) used was an API 4000 triple quadrupole (Applied Biosystems). A calibration curve was obtained running standard synthetic AHLs (with and without oxo- and hydroxy-substitutions, and ranging from 4 to 18 carbons in their acyl chains). The calibration curve was used to quantify AHLs in the samples. Elution times and molecular spectra of the synthetic standard AHLs was used to identify the AHLs present in the samples (Romero et al., 2010; Mayer et al., 2018).

15. Confocal-laser scanning microscopy imaging

15.1. Subgingival polymicrobial biofilms

Subgingival biofilms grown in McBain and anaerobiosis in the AAA model, generated as described in section 9.1.1, were analysed by confocal microscopy to compare control and Aii20J-treated conditions. Biofilms formed onto the glass coverslips were stained with SYTO 9 and PI (LIVE/DEAD BacLight Bacterial Viability Kit, Thermo Fisher Scientific). The staining solution was prepared by mixing SYTO 9 (3.34 mM) and PI (20 mM) in a 1:1 proportion and further diluting it 150-times in sterile PBS (pH 7.4), to final working

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concentrations of 11 μM for SYTO 9, and 66 μM for PI. Fifty microliters of the staining solution were poured onto each sample, and incubated for 15 minutes at RT, protected from light, before image acquisition with a Leica Stellaris confocal microscope (Leica Microsystems GmbH, Wetzlar, DE). The excitation/emission wavelengths were 480/500 nm for SYTO 9 and 490/635 nm for PI. SYTO 9 is membrane permeable and enters all cells regardless of membrane integrity, while PI only penetrates membrane-damaged bacterial cells.

Microphotographs of the biofilms were taken at 20x magnification. To calculate the area covered by the biofilms, 8 randomly selected fields were analysed for each condition with the Leica Application Suite X software (LAS X v.3.7.4, Leica Microsystems). To do so, the area which received photons on both the 530 nm channel (cells stained with SYTO 9) and 622 nm channel (cells stained with PI) was divided by the total area. The results describe in percentage the area covered by the biofilm.

15.2. Monospecies E. faecalis biofilms

E. faecalis biofilms were generated as previously described in section 9.2, in the presence the short- and long-chain AHL mixes, as well as of C_{18}-HSL, onto polystyrene discs of 16 mm of diameter that were placed in the bottom of 24-well polystyrene plates. After 6 hours of incubation, biofilms were washed three times with PBS and immediately stained with SYTO 9 and PI (LIVE/DEAD BacLight Bacterial Viability Kit, Thermo Fisher Scientific), following the methodology described above (section 15.1).

Microphotographs of the biofilms were taken at 20x and 40x magnification. To calculate the area covered by the biofilms, 8 randomly selected fields taken at 20x were analysed for each condition with the Leica Application Suite X software (LAS X v.3.7.4, Leica Microsystems), as described in section 15.1. The results describe in percentage the area covered by *E. faecalis* biofilms.

16. *In silico* search of QS- and QQ-related sequences in oral metagenomes

A selection of QS and QQ-related sequences was made by manually searching NCBI's databases (https://www.ncbi.nlm.nih.gov/) for annotated nucleotide and amino acid sequences with demonstrated activity. Selected QS sequences comprised AHL synthases (n = 36), AHL receptors (n = 27), and the AI-2 synthase LuxS (n = 11). Selected QQ sequences included three types of enzymes: lactonases (n = 63), acylases (n = 20), and paraoxonases (n = 3). Nucleotide

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sequences were translated into amino acid sequences using the software Expasy (Duvaud et al., 2021).

The initial 160 sequences were clustered into protein families using CD-HIT (Fu et al., 2012), establishing a minimum of 70% length coverage and 40% sequence identity to reduce redundancy. Even though several protein sequences have the same annotation (e.g., LuxS), some were clustered into different protein families, reflecting evolutionary divergence (e.g., LuxS sequences were clustered into 4 groups with the representative type sequences corresponding to *V. harveyi, Streptococcus pyogenes, Bacillus thuringiensis,* and *Bacteroides heparinolyticus*). The 160 sequences were clustered and grouped by function, resulting in 68 protein families. The accession numbers the sequences contained in each protein family are available in Supplementary Table S1 (Supplementary_Table_S1.pdf).

The representative sequences selected for each protein family were then used to query 118 shotgun oral metagenomes from the Human Microbiome Project (Human Microbiome Project Consortium, 2012a, 2012b) (https://portal.hmpdacc.org/) (available in Supplementary Table S2; Supplementary_Table_S2.pdf) and 7 oral metagenomes from a Spanish collection (Belda-Ferre et al., 2012). All 125 metagenomes corresponded to supragingival plaque samples.

Sequence searches were performed using DIAMOND (Buchfink et al., 2021) with a minimum subject coverage of 70% and an e-value cut-off of 1e-10. Alignment results were parsed into tables using in-home scripts from the National Autonomous University of Mexico (MX) and are available at https://github.com/genomica-fciencias-unam/tabla_genes. Statistical analyses were done using R (R Core Team, 2022) with the phyloseq (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2009), vegan (Oksanen et al., 2017), and tydiverse (Wickham et al., 2019) packages.

17. Statistical analyses

17.1. Polymicrobial oral biofilms

Statistical analyses were performed using GraphPad Prism 8.3.0 (GraphPad, San Diego, CA, USA, www.graphpad.com) or R (R Core Team, 2022). The datasets fit to a normal distribution was tested by Shapiro-Wilk tests. Two-tailed Student's *t*-tests (referred to in the text as *t*-tests) for normally distributed samples and Mann-Whitney tests for non-normally distributed samples were performed to determine the statistical significance of the differences between control and Aii20J-treated biofilm mass and cell index values within the same donor.

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In addition, Wilcoxon matched-pairs signed-rank tests (referred to in the text as Wilcoxon tests) were performed to assess the significance of the reductions in biofilm formation in the presence of Aii20J throughout samples originated from different patients and grown in the same conditions. ANOVA tests were used to compare more than two groups of samples. Quantitative PCR results were analysed using Mann-Whitney tests for independent samples. For all the statistical analyses, significant differences were determined with an $\alpha = 0.05$.

17.2. Monospecies E. faecalis biofilms

Statistical analyses were performed using GraphPad Prism 8.3.0 (GraphPad, San Diego, CA, USA, www.graphpad.com). The datasets fit to a normal distribution was tested by Kolmogorov-Smirnov or Shapiro-Wilk tests as appropriate. Accordingly, pairwise comparisons were analysed using either Student's *t*-tests or Mann-Whitney tests, and ANOVA or Kruskal-Wallis tests were used to assess statistical significance between more than two groups. Significance was set at $\alpha = 0.05$ for all analyses.

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Results and Discussion

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CHAPTER 1 | Inhibition of periodontal biofilms *in vitro* using Quorum Quenching

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The contents of this chapter make part of a research article titled "The quorum quenching enzyme Aii20J modifies *in vitro* periodontal biofilm formation", authored by Ana Parga[1], Andrea Muras[1], Paz Otero-Casal[2,3], Alexandre Arredondo[4], Agnès Soler-Ollé[4], Gerard Àlvarez[4], Luis D. Alcaraz[5], Alex Mira[6], Vanessa Blanc[4], and Ana Otero[1].

The research article has been submitted to *Frontiers in Cellular and Infection Microbiology* and it does not have an identifier yet.

[1] Department of Microbiology and Parasitology, CIBUS-Faculty of Biology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
[2] Department of Surgery and Medical-Surgical Specialties, Faculty of Medicine and Odontology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
[3] Unit of Oral Health, CS Santa Comba-Negreira, SERGAS, Spain
[4] Department of Microbiology, Dentaid Research Center, Cerdanyola Del Vallès, Spain
[5] Department of Cellular Biology, Science Faculty, National Autonomous University of Mexico, Coyoacán, Mexico
[6] Department of Genomics and Health, FISABIO Foundation, Valencia, Spain

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Background

Most oral bacteria are embedded in a matrix of extracellular polymers of host and bacterial origin and attached to the tooth surface forming a biofilm, presenting altered phenotypes compared with planktonic cells (Donlan and Costerton, 2002; Marsh, 2006). The two major oral bacterial diseases, dental caries and periodontal disease (PD) are caused by changes in local environmental conditions that result in a loss of the ecological equilibrium in the oral microbiota, eventually leading to a pathogenic biofilm (Marsh, 1994, 2003; Belda-Ferre et al., 2012; Hajishengallis et al., 2012). It is not a single pathogen but rather the entire community residing in the oral cavity and its functional activities that are responsible for the progression of these diseases (Duran-Pinedo and Frias-Lopez, 2015; Yost et al., 2015). In this context, interfering with biofilm formation processes through modulation of microbial interactions has been proposed as a relevant strategy for preventing and treating oral polymicrobial diseases (Simón-Soro and Mira, 2015).

Cell density-dependent gene-regulation processes, known as quorum sensing (QS), have been proposed as potential targets for developing strategies to inhibit oral biofilm formation (Muras et al., 2018, 2020b; Muras and Otero, 2020). Among the three most studied QS signals, the presence of gram-positive autoinducer peptides has been reported in various oral streptococci, while the universal autoinducer-2 (AI-2) has been found in both gram-positive and gram-negative oral pathogens (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Kolenbrander et al., 2006). The *N*-acyl-homoserine lactones (AHLs), the QS signals typically used by gram-negative bacteria, have traditionally been considered non-relevant in the oral cavity due to the lack of success in detecting their production in pure cultures of oral pathogens in the past (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Jakubovics and Kolenbrander, 2010; Huang et al., 2011b; Guo et al., 2014). Despite this, a diversity of AHLs has been found in saliva samples and extracted teeth, including C_8-HSL, C_{14}-HSL, and C_{18}-HSL (Kumari et al., 2006; Muras et al., 2020b). Additionally, AHL-producing bacterial strains have been isolated from dentine caries (Goh et al., 2014, 2016) and human tongue surface (Yin et al., 2012b, 2012a; Chen et al., 2013). The production of OC_8-HSL has been described in pure cultures of the periodontopathogen *Porphyromonas gingivalis* (Muras et al., 2020b). Previous studies had observed that AHLs and AHL analogues modified protein expression in *P. gingivalis* (Komiya-Ito et al., 2006; Asahi et al., 2010). Moreover, a recent study on the effect of exogenous AHLs on *in vitro* oral biofilms proved that certain AHLs

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affected bacterial lactic acid production and protease activity and that the addition of C_6-HSL shifted the microbial composition of biofilms towards a periodontal bacterial profile (Muras et al., 2020a). In addition, bacterial gene clusters predicted to be related to homoserine lactone biosynthesis have been described in the genomes of bacteria from caries and periodontitis patients (Aleti et al., 2019). Together, these results suggest that the established role of AHLs in the current paradigm of cell-to-cell communication in oral biofilms should be revised (Muras and Otero, 2020; Muras et al., 2020b, 2022). Confirmation of the role of AHLs as QS signals in oral biofilm formation would open a broad range of possibilities for the prevention and treatment of oral diseases.

Different strategies to interfere with QS systems are present in natural environments. The use of these strategies in both natural and *in vitro* conditions has been successful in decreasing biofilm mass in single-species (Sun et al., 2021) and multispecies biofilms (Jo et al., 2016; Huang et al., 2019; Schwab et al., 2019; Jeong et al., 2020; Muras et al., 2020b, 2022). The use of these strategies is less likely to promote antimicrobial resistance than with antibiotics, as they do not interfere directly with bacterial growth (Romero et al., 2014, 2015; Mayer et al., 2015, 2018; García-Contreras et al., 2016; Guendouze et al., 2017; Muras et al., 2018). The enzymatic inactivation of QS signals, also known as quorum quenching (QQ), has been thoroughly studied (Romero et al., 2015), especially enzymes capable of disrupting AHLs (Dong et al., 2001). Remarkably, the presence of QQ activity against AHLs has already been described in cultivable bacteria from saliva and dental plaque samples (Muras et al., 2020b). The use of Aii20J, a broad-range, highly active AHL-lactonase belonging to the metallo-beta-lactamase family (Mayer et al., 2015), has yielded interesting results for controlling several AHL-regulated phenotypes in gram-negative pathogenic bacteria (Mayer et al., 2015, 2018, 2020) and reducing oral biofilm grown *in vitro* from saliva (Muras et al., 2020b).

To gain fundamental knowledge on oral biofilm formation, growth dynamics, structure, antimicrobial tolerance and host-pathogen interactions, the scientific community has greatly relied on laboratory biofilm models. *In vivo* animal models provide useful information on the relationship between biofilm and oral disease. However, these models are expensive and entail several ethical implications. Hence, a vast diversity of *in vitro* biofilm models have been developed. The design of *in vitro* models to study oral biofilms takes into consideration several factors; the reproducibility and cost-effectiveness of the system, the flexibility to adapt culture conditions to the particularities of the oral environment, and the maintenance of interactions

within the microbial community (Ouidir et al., 2022). To reproduce the oral environment, models that allowed the growth of biofilms under constant flow and exposed to shear forces were developed. These models use a chemostat flow system that constantly renews the culture media (Brown et al., 2019). These systems were used to describe interspecies interactions that were close to the ones occurring *in vivo* in the oral biofilm and were also used to test the antimicrobial susceptibility of biofilms (McDermid et al., 1987; Bradshaw et al., 1990, 1993). The low throughput of the chemostat models was decisive for many research groups to use static models that allowed high-throughput screening for susceptibility to antimicrobials, as well as phenotypic characterisation of mutant libraries (Thrower et al., 1997; Wright et al., 1997; Loo et al., 2000). In static models, biofilms can be grown onto different substrates, such as plastic, glass, or hydroxyapatite (HA), directly onto the bottom of microtiter plates or on inverted pegs submerged in culture media with a specific inoculum, i.e., the Calgary Biofilm Device (Ceri et al., 1999). One of the disadvantages associated with static models is the quantification of both adhered and sedimented cells if biofilms are not properly washed before their quantification or characterisation. To overcome this limitation, in the Academic Centre for Dentistry in Amsterdam (ACTA), an active adhesion model was developed: the Amsterdam Active Attachment model (AAA model) (Exterkate et al., 2010). This static model comprises the use of a modified microtiter plate lid with the desired substrate fixed in designated positions so that upon closing the plate, the substrate is vertically submerged in a well, containing culture medium and the inoculum (Exterkate et al., 2010). The AAA model allows the refreshment of the culture media, which further enhances the growth of the attached bacteria, as it eliminates the competition with the planktonic cells, and ultimately results in the generation of sufficient biofilm mass to perform imaging studies or nucleic acid or protein extractions to further investigate biofilms.

Recently, modern technologies for real-time monitoring of cellular growth have been developed. Specifically, the xCELLigence Real-Time Cell Analyser (RTCA) System (ACEA Biosciences) has been validated for the study of bacterial biofilms (Gutiérrez et al., 2016; Ferrer et al., 2017; Muras et al., 2018) and, particularly, oral biofilms (Mira et al., 2019). This system is based on impedance measurements registered by electronic sensors placed on the bottom of dedicated plates (E-plates, ACEA Biosciences). Thus, this system measures only the biofilm formed onto the bottom of the well.

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Another important consideration when establishing an experimental design setting is the choice of single or multiple species for the generation of biofilms. Single-species models allow more precise mechanistic studies and have been key for establishing fundamental knowledge of biofilm biology. This approach is still chosen nowadays for different purposes, such as the characterisation of biofilm dynamics (Abrantes and Africa, 2020; Martinez et al., 2020) and the investigation of antibiofilm compounds (Gutiérrez et al., 2016; Ferrer et al., 2017; Muras et al., 2018; Loimaranta et al., 2020; Žiemytė et al., 2020, 2021). To increase the complexity of the biofilm models, and thus to represent more faithfully the oral cavity environment, researchers aimed to develop models comprising a higher number of species. The use of dual-species models has been extremely useful in studying interactions between oral species (Willems et al., 2016; Philip et al., 2019), but with the spread of widely available real-time quantitative polymerase chain reaction (RT-qPCR) equipment, the development of even more complex models was achieved. Monitoring biofilm composition with specific probes and RT-qPCR permitted the reproducible growth of biofilms of very precise composition. It is worth highlighting here the initial development of a five-species model in Zürich (Guggenheim et al., 2001) that has been continuously optimised, reaching the state of ten-species model (Ammann et al., 2013) that serves as a base for customised variants to study several oral diseases (Thurnheer and Belibasakis, 2016; Bao et al., 2018). A highly complex but very stable 14-species model was obtained by Slomka et al. (2018) by using a chemostat.

The last level of complexity regarding microbial communities in *in vitro* oral biofilm models is the use of microcosm models, in which oral samples are used as inocula. These models have accomplished the generation of biofilms with a number of taxa that would not be achievable using any of the previously commented methodologies. Several research groups have implemented microcosm biofilm models using saliva as inoculum, with a special interest in the use of these models to test the efficacy of antimicrobial strategies (Caldeirão et al., 2021; Exterkate et al., 2022; Mao et al., 2022). Microcosm models have also been used to assess the effect of prebiotics (Janus et al., 2017) and strategies targeting cell-cell signalling in bacteria (Janus et al., 2016; Muras et al., 2020a, 2020b). The use of a defined consortium of bacteria results in a more reproducible approach to studying oral biofilms. However, next-generation sequencing techniques have revealed how microcosm models can achieve bacterial communities more similar to *in vivo* biofilms, as far as culture conditions are carefully selected (Janus et al., 2015). Microcosm models also allow the assessment of biofilm responses to

antimicrobial agents while accounting, to a certain extent, for interindividual differences (Sánchez et al., 2021). The biofilm model of choice will then depend on the aims of each particular study.

In this work, we examined the effect of Aii20J on *in vitro* biofilms derived from saliva and subgingival samples obtained from healthy donors and patients with PD using different biofilm formation and quantification techniques and different culture conditions. We describe a significant reduction in biofilm mass due to Aii20J that does not affect the microbial relative abundance of the biofilms.

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Results and discussion

1. Sample origin and biofilm generation systems influence *in vitro* oral biofilm formation and sensitivity to the AHL-lactonase Aii20J

To assess the potential use of Aii20J in the prevention of oral diseases, the biofilm inhibitory capacity of the enzyme was first explored using two cultivation techniques: the AAA model (Exterkate et al., 2010; Muras et al., 2020a) and the xCELLigence system (ACEA, Biosciences Inc.). Both systems were inoculated with saliva and subgingival samples from seven healthy donors (codes H1 – H7) and ten patients with periodontal disease (PD) (codes P1 – P10) (see Table 3 in the Methods section). Samples were grown aerobically in Brain-Heart Infusion (BHI) medium and anaerobically in Schaedler culture medium supplemented with vitamin K1 (0.1 mg/L) (SCH-K1). We observed variations in total attached biomass among samples (Figures 6 and 7), without significant differences in biomass values achieved by healthy and PD groups grown in the AAA model (*t*-test) (Figure 6A,B,C,D). In the xCELLigence system, PD samples yielded significantly higher cell index values than healthy samples for subgingival biofilms grown in BHI and saliva biofilms grown in SCH-K1 (*t*-test) (Figure 7A,D). Last, initial biofilm mass achieved in the control condition did not correlate with sensitivity to Aii20J in any cultivation system (Pearson r) (data not shown).

When cultivated in the AAA model, subgingival biofilms were more sensitive to Aii20J than saliva biofilms in both culture media used (Figure 8). Analysis of the effect of the enzyme on subgingival biofilms grouped according to the oral health status of the subjects showed significant biomass reductions in PD-derived biofilms grown in BHI and in both healthy and PD samples grown in SCH-K1 (Wilcoxon test) (Figure 8A,B). In subgingival, PD-derived biofilms, macroscopical differences were spotted in 60% of the samples grown in BHI and 44% of samples grown in SCH-K1. As assessed with CV staining, biofilm biomass was significantly reduced in 40% of biofilms grown in BHI and 33% of biofilms grown in SCH-K1 (*t*-test) (Figure 8A,B). Additionally, subgingival, healthy-derived biofilms presented macroscopical differences in 71% of samples grown in BHI and 57% of samples grown in SCH-K1, and significant biofilm biomass reductions were reported for 14% of biofilms grown in BHI, and 57% of biofilms grown in SCH-K1 (*t*-test) (Figure 8A,B).

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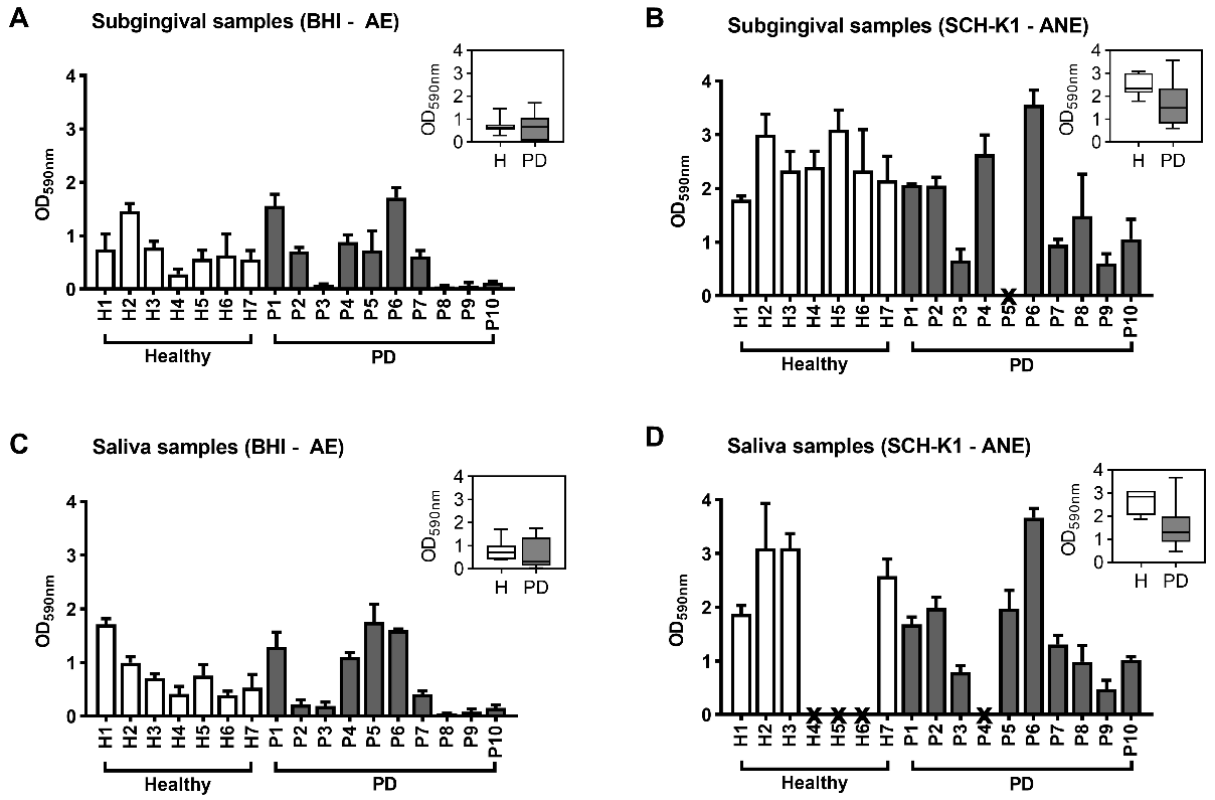


Figure 6. Biofilm formation in the AAA model, represented as mean ± SD (*n* = 3) of control biofilms (OD_{590nm}). Samples used to inoculate the biofilms were obtained from healthy donors (white bars) and patients with periodontal disease (PD, grey bars). All biofilms were grown for 24 hours. Subgingival samples were grown in BHI and aerobiosis (A) **and SCH-K1 and anaerobiosis (B). Saliva samples were grown in BHI and aerobiosis (C) and SCH-K1 and anaerobiosis (D). In samples P5 (B) and H4, H5, H6 and P4 (D), marked with an "X" on the x-axis, biofilm could not be quantified. Inserted in each graph is a boxplot (median and interquartile range -IQR-, whiskers range from minimum to maximum) comparing absolute values of control biofilms (OD_{590nm}) from healthy (H) donors (*n* = 7) and patients with periodontal disease (PD) (*n* = 10). Asterisks (*) indicate statistical significance (t-tests, *α = 0.05). AE: aerobiosis. ANE: anaerobiosis. Reproduced with permission from Parga et al. (submitted).**

Saliva samples were cultured in the same conditions in the AAA model. When analysing the enzyme's effect on saliva biofilms based on the oral health status of the donors, only PD-derived biofilms grown in SCH-K1 experienced a significant reduction in biomass (Wilcoxon test) (Figure 8C,D). Regarding PD-derived saliva biofilms, macroscopical differences due to Aii20J were described for 40% of biofilms grown in BHI and 44% of biofilms grown in SCH-K1. Significant reductions in Aii20J-treated samples were reported for 10% BHI biofilms and 56% SCH-K1 biofilms (*t*-test) (Figure 8C,D). In healthy-derived saliva biofilms, macroscopical differences were detected in 71% of biofilms grown in BHI, and biomass reduction was

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statistically significant in 43% of BHI biofilms (*t*-test) (Figure 8C). When saliva from healthy donors was inoculated in SCH-K1, almost half of the biofilm samples detached from the coverslip when washing the CV staining, both in control and Aii20J-treated samples, impeding their correct quantification. No significant differences were observed for those biofilms that could be measured (*t*-test) (Figure 8D). The observation of different responses to the Aii20J enzyme between biofilms generated from subgingival samples and saliva could be explained by the variation of bacterial communities depending on the sample used as inoculum, which has already been reported (Simón-Soro et al., 2013; Frias-Lopez and Duran-Pinedo, 2020).

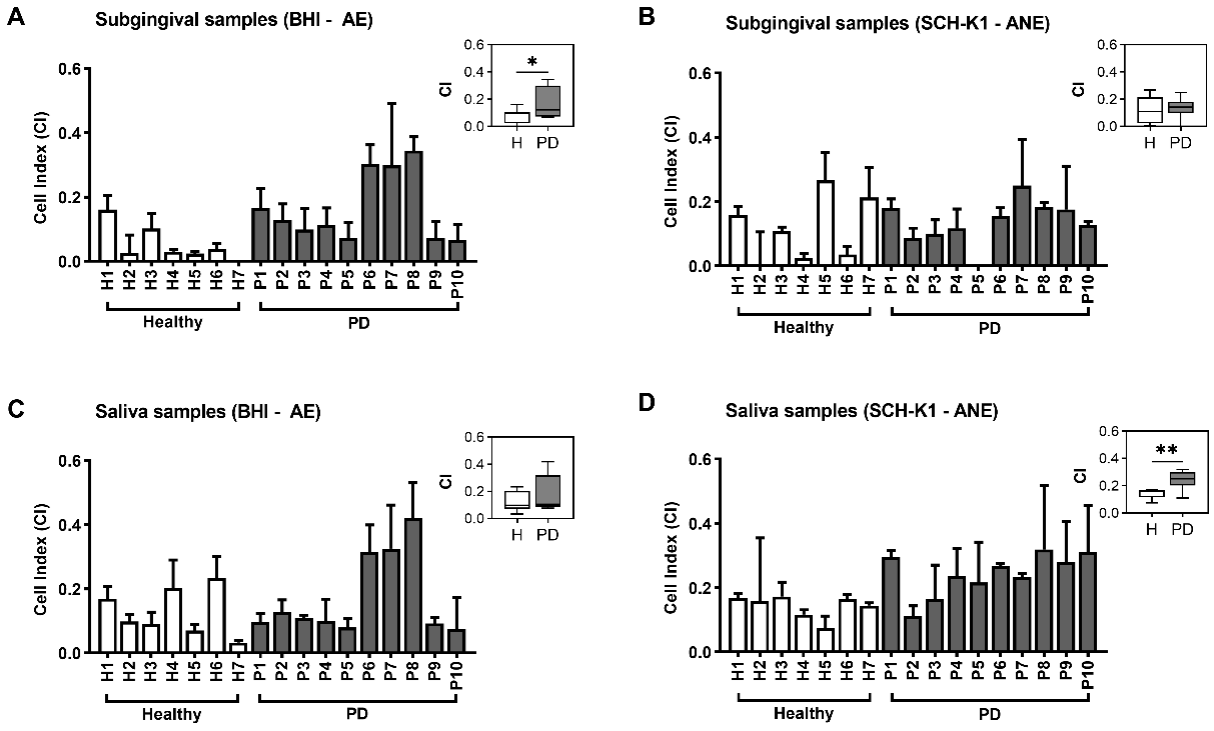


Figure 7. Biofilm formation in the xCELLigence system at 24 hours of incubation. Results are represented as mean ± SD (*n* = 3) cell index (CI) values of control biofilms. Samples used to inoculate the biofilms were obtained from healthy donors (white bars) and patients with periodontal disease (PD, grey bars). Subgingival samples were grown in BHI and aerobiosis (A) and SCH-K1 and anaerobiosis (B). Saliva samples were grown in BHI and aerobiosis (C), and SCH-K1 and anaerobiosis (D). Inserted in each graph is a boxplot (median and interquartile range -IQR-, whiskers range from minimum to maximum) comparing absolute values of control biofilms (CI) from healthy (H) donors (*n* = 7) and patients with periodontal disease (PD) (*n* = 10). Asterisks (*) indicate statistical significance (*t*-tests, *α = 0.05, **α = 0.01). AE: aerobiosis. ANE: anaerobiosis. Reproduced with permission from Parga et al. (submitted).

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The xCELLigence system was much less sensitive than the AAA model in detecting the effect of Aii20J, as no significant changes were found in subgingival samples treated with the enzyme (*t*-test) (Figure 9A,B). In only two cases, Aii20J-treated saliva samples showed significant reductions compared with their controls when cultured in this system, either in BHI or SCH-K1 (*t*-test) (Figure 9C,D). Altogether, our results indicate that the AAA model is a more sensitive methodology than the xCELLigence system for detecting different responses of biofilms to Aii20J. The AAA model allows quantification, macroscopic evaluation (see Figure 5 in the Methods section), and microscopy imaging of the biofilms attached to the glass coverslips, complementing the CV assay for detecting structural differences (Muras et al., 2018). The AAA model also facilitates the refreshment of treatments and culture media, making more nutrients available for the attached cells, eliminating loose bacteria and metabolic waste, and ensuring the assessment of actively attached cells only. On the other hand, the xCELLigence system monitors biofilm growth during early adhesion stages (Muras et al., 2018; Mira et al., 2019), reaching saturation after 10 hours of incubation (Muras et al., 2018). In the AAA model, an end-point quantification technique, we describe significant biofilm alterations due to Aii20J. In contrast, in the xCELLigence system, a real-time monitoring technology, no significant changes were found for most samples. Thus, Aii20J may affect biofilm in its maturing phase rather than in the initial attachment stages. Since the AAA model allowed the generation of biofilms in which more differences were observed after treatment with Aii20J, this biofilm cultivation model was selected for further experiments.

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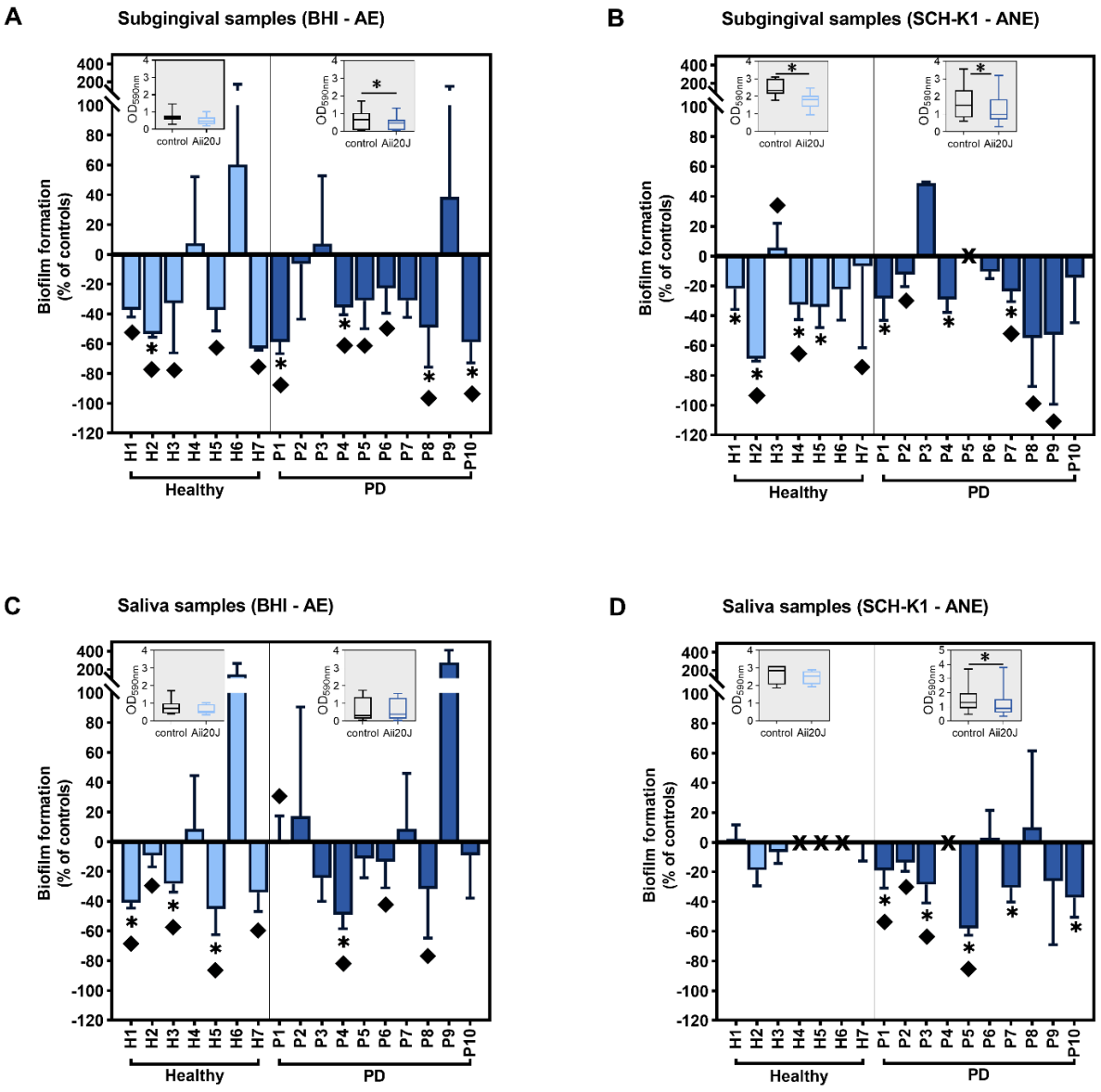


Figure 8. Inhibition of saliva and subgingival biofilm formation in the AAA model in the presence of Aii20J. Results are represented as mean ± SD (*n* = 3) of the percentage of biomass in Aii20J-treated biofilms relative to biomass in untreated controls (OD_{590nm}). Samples used to inoculate the biofilms were obtained from healthy donors (light blue bars) and patients with periodontal disease (PD; dark blue bars). All biofilms were grown for 24 hours. Subgingival samples were grown in BHI and aerobiosis (A) and SCH-K1 and anaerobiosis (B). Saliva samples were grown in BHI and aerobiosis (C) and SCH-K1 and anaerobiosis (D). Asterisks (*) indicate statistical significance (*t*-tests, *α = 0.05). Diamonds (♦) represent macroscopical differences between untreated and Aii20J-treated biofilms. In samples P5 (B) and H4, H5, H6 and P4 (D), marked with an "X" on the x-axis, biofilms could not be quantified. Inserted in each graph is a boxplot (median and interquartile range -IQR-, whiskers range from minimum to maximum) comparing absolute values of biofilm mass (OD_{590nm}) in control and Aii20J-treated samples from healthy donors (*n* = 7) and PD patients (*n* = 10). Asterisks (*) indicate statistical significance (Wilcoxon tests, *α = 0.05). AE: aerobiosis. ANE: anaerobiosis. BHI: Brain Heart Infusion. SCH-K1: Schaedler medium supplemented with 0.1 mg/L of vitamin K1. Reproduced with permission from Parga et al. (submitted).

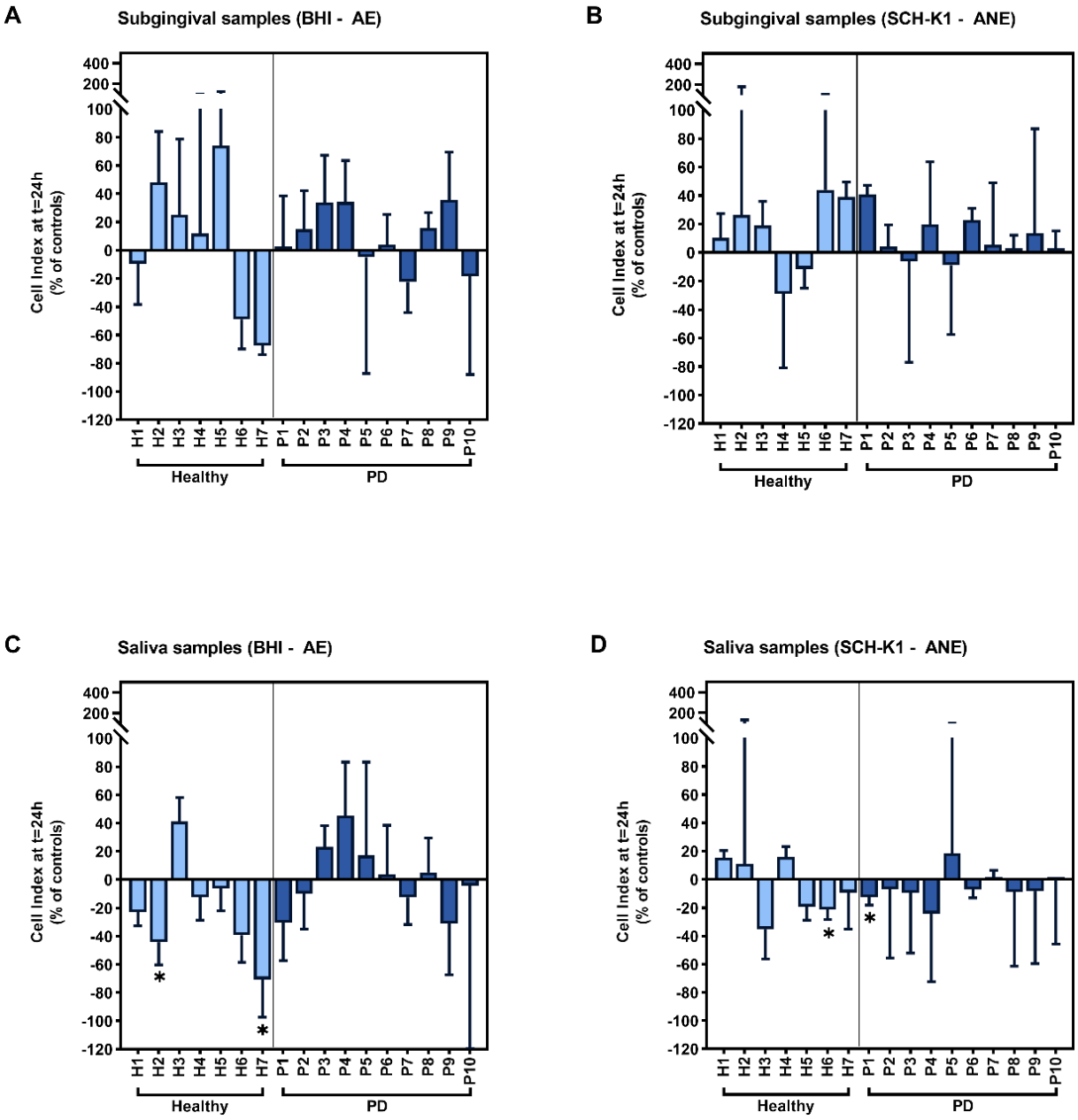


Figure 9. Inhibition of biofilm formation in the xCELLigence system in the presence of Aii20J at 24 hours of incubation. Results are represented as mean ± SD (n = 3) of the percentage of cell index values of Aii20J-treated biofilms relative to biomass in their untreated control. Samples used to inoculate the biofilms were obtained from healthy donors (light blue bars) and patients with periodontal disease (PD, dark blue bars). Subgingival samples were grown in BHI and aerobiosis (A) and SCH-K1 and anaerobiosis (B). Saliva samples were grown in BHI and aerobiosis (C) and SCH-K1 and anaerobiosis (D). Asterisks (*) indicate statistical significance (t-tests, *α = 0.05). AE: aerobiosis. ANE: anaerobiosis. Reproduced with permission from Parga et al. (submitted).

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Additionally, we evaluated the prevalence of QQ activity among the initial saliva samples with standardised bioassays (Romero et al., 2011) against short- (C_6-HSL) and long-chain (C_{12}-HSL) AHLs. QQ activity against C_{12}-HSL was common, with five out of seven saliva samples from healthy donors and seven out of eight saliva samples of PD origin inhibiting AHL detection by the biosensor strain VIR07 (Figure 10). Conversely, QQ against C_6-HSL was scarce, with only two healthy and one PD saliva samples decreasing violacein production in the biosensor strain CV026 (Figure 10). These observations confirm previous reports on QQ activity being more abundant in samples from a periodontitis patient than in a healthy patient (Muras et al., 2020b), suggesting a more relevant role of AHLs in PD subjects. However, a higher number of samples would be needed to confirm the significance of this finding.

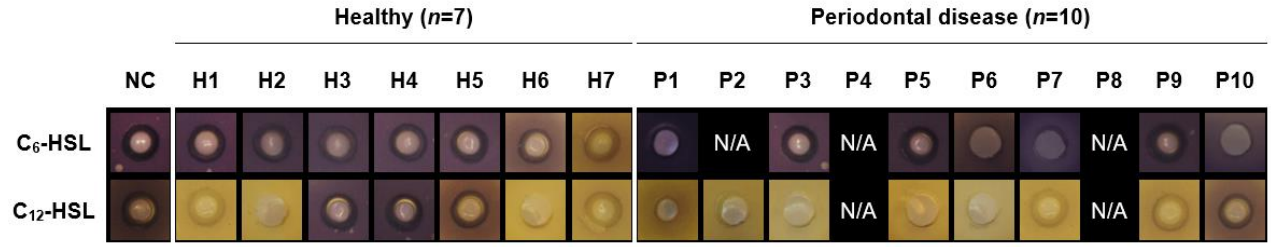


Figure 10. QQ activity in saliva samples. A violacein halo around the wells after 24 hours of incubation with C6-HSL or C12-HSL indicates that the AHL preserves its original structure. Reduced or inexistent halos show partial and total degradation of the AHL, respectively. NC: negative control. N/A: not available. Reproduced with permission from Parga et al. (submitted).

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2. Culture conditions driving the generation of *in vitro* oral biofilms with high microbial diversity

To assess which culture media and incubation conditions resulted in higher microbial diversity within the *in vitro* biofilms, a wider array of culture conditions was explored using subgingival samples, as they are supposed to be more representative of the periodontal microbiota than saliva samples. Samples were taken from a healthy donor (H8), a patient with gingivitis (G1), and two patients with periodontitis (P11, P12). Subgingival biofilms were generated in the AAA model in four chosen conditions: BHI-aerobiosis, BHI-anaerobiosis, SCH-K1-anaerobiosis, and McBain-anaerobiosis, to select the conditions driving the generation of biofilms more representative of a periodontal biofilm (Figure 11).

Biofilm formation was heterogeneous among the culture media and conditions tested, with the higher biomass values achieved in SCH-K1 (Figure 11A). The treatment of these biofilms with the QQ enzyme resulted in macroscopical differences (see Figure 5, in Methods section) in 75 – 100% of the patients compared with the untreated biofilms (Figure 11A). Significant reductions of subgingival-derived biofilm mass were observed for 50 – 75% of patients among culture conditions (Figure 11A).

Microbial community profiling, achieved by sequencing the 16S rRNA gene, revealed that, regardless of the incubation conditions, the genus *Streptococcus* was predominant in all biofilms (Figure 11B). However, McBain medium, containing a higher protein load than the other culture media, allowed the proliferation of more diverse communities, with increased proportions of genera such as *Veillonella, Granulicatella, Gemella,* and *Haemophilus* both in control and Aii20J-treated biofilms, as compared with the other culture media. These conditions (the use of McBain medium in anaerobiosis) have already been proposed as an adequate option to recreate the gingival crevice niche *in vitro* (Janus et al., 2015), as high availability of nutrients and low oxygen conditions have been described to favour the proliferation of asaccharolytic and proteolytic bacteria *in vivo* (Drucker, 2000). Worth mentioning here is that the described significant reductions in biofilm mass obtained with the use of Aii20J occurred even in the gram-positive predominated biofilms obtained in BHI and SCH-K1 media. Nevertheless, these reductions in biofilm mass occurred without significant changes in bacterial relative abundance at the genus level (Figure 11A,B).

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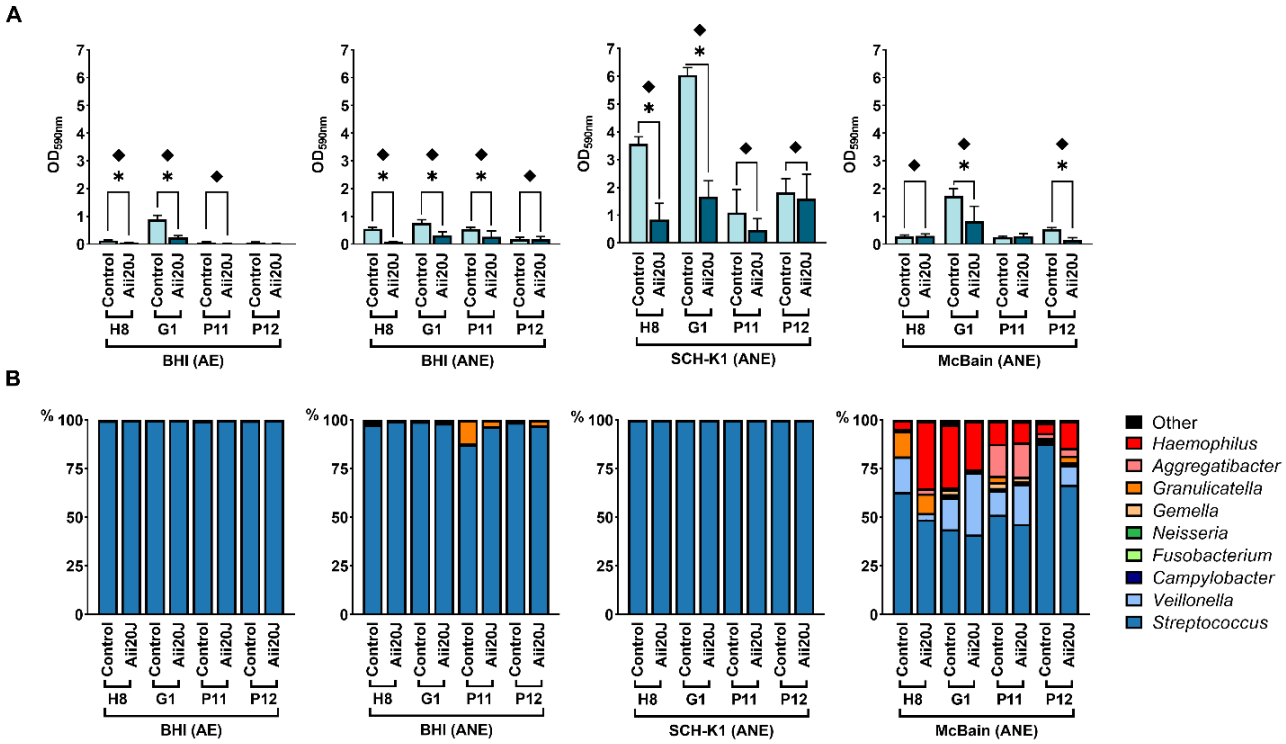


Figure 11. Effect of culture media and conditions on *in vitro* subgingival-derived biofilm formation (A) and bacterial diversity (B) in the presence of Aii20J. Subgingival samples from four patients were grown in the AAA model for 24 hours in four different culture conditions: BHI in aerobiosis, and BHI, SCH-K1, and McBain medium in anaerobiosis. A. Biofilm formation is represented as mean ± SD (*n* = 3) of control and Aii20J-treated biofilms absorbance values (OD_{590nm}). Asterisks (*) indicate statistical significance (*t*-tests, *α = 0.05). Samples marked with a diamond (♦) presented macroscopical differences between untreated and Aii20J-treated biofilms. B. Relative abundance (in percentage) of the most prevalent genera in the biofilms, as assessed by 16S rRNA gene sequencing. Genera detected in lower relative abundance appear under the category "Other". AE: aerobiosis. ANE: anaerobiosis. Reproduced with permission from Parga et al. (submitted).

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3. Effect of the AHL-lactonase Aii20J on *in vitro* subgingival-derived biofilm formation and bacterial diversity

Once we established the optimal conditions for obtaining diverse oral biofilms in the AAA model, we studied a greater number of samples, assessing changes in biofilm mass and microbial diversity in the presence of Aii20J. Subgingival samples were chosen as inocula since they presented higher sensitivity to Aii20J than saliva samples, as seen in previous experiments (see Figure 8), and were more representative of the periodontal microbiota (see Figure 11). For subsequent experiments, 31 patients, in addition to the four patients previously studied, were recruited, comprising a total of 11 healthy donors (H8 – H18), three patients with gingivitis (G1 – G3), and 21 patients with periodontitis (P11 – P31) (see Table 4 in the Methods section). Biofilms were grown in BHI-anaerobiosis and McBain-anaerobiosis to compare the effect of Aii20J on biofilms dominated by gram-positive bacteria and on more diverse biofilm communities, respectively. As seen with the initial set of patients, biofilm mass values obtained in the control condition were widely varied (Figure 12). No significant differences in biomass values between healthy and PD groups were observed (*t*-test) (Figure 12). Besides, biomass values of untreated biofilms did not correlate with sensitivity to Aii20J (Pearson r) (data not shown).

Overall, biomass was reduced in the presence of Aii20J in both biofilms grown in BHI-anaerobiosis and McBain-anaerobiosis, with few exceptions (Figure 13), confirming previous results in *Streptococcus*-dominated biofilms (see Figure 8). When the effect of Aii20J was analysed by grouping the biofilms according to the oral health classification of the patients, PD-derived biofilms were significantly reduced in all culture conditions, while healthy-derived biofilms were significantly reduced only when grown in BHI (Wilcoxon test) (Figure 13). In subgingival, PD-derived biofilms, macroscopical differences (see Figure 5, in Methods section) were detected in 71% of biofilms grown in BHI and 38% of biofilms grown in McBain in the presence of Aii20J, and biomass reduction was statistically significant in 50% and 42% of the samples, respectively (*t*-test) (Figure 13). In subgingival, healthy-derived biofilms, macroscopical differences were found in 91% of biofilms grown in BHI and 18% of biofilms grown in McBain. Additionally, biomass reduction due to Aii20J was significant in 64% of healthy biofilms grown in BHI and 18% of healthy biofilms grown in McBain (*t*-test) (Figure 13). Assessment of additional controls, i.e., the fraction smaller than 10 kDa present in the purified Aii20J solution, and the heat-inactivated Aii20J, yielded no differences in biofilm mass

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compared with water-only negative controls (Figure 14). These results further confirm the effectiveness of this enzyme as an *in vitro* biofilm inhibitory strategy.

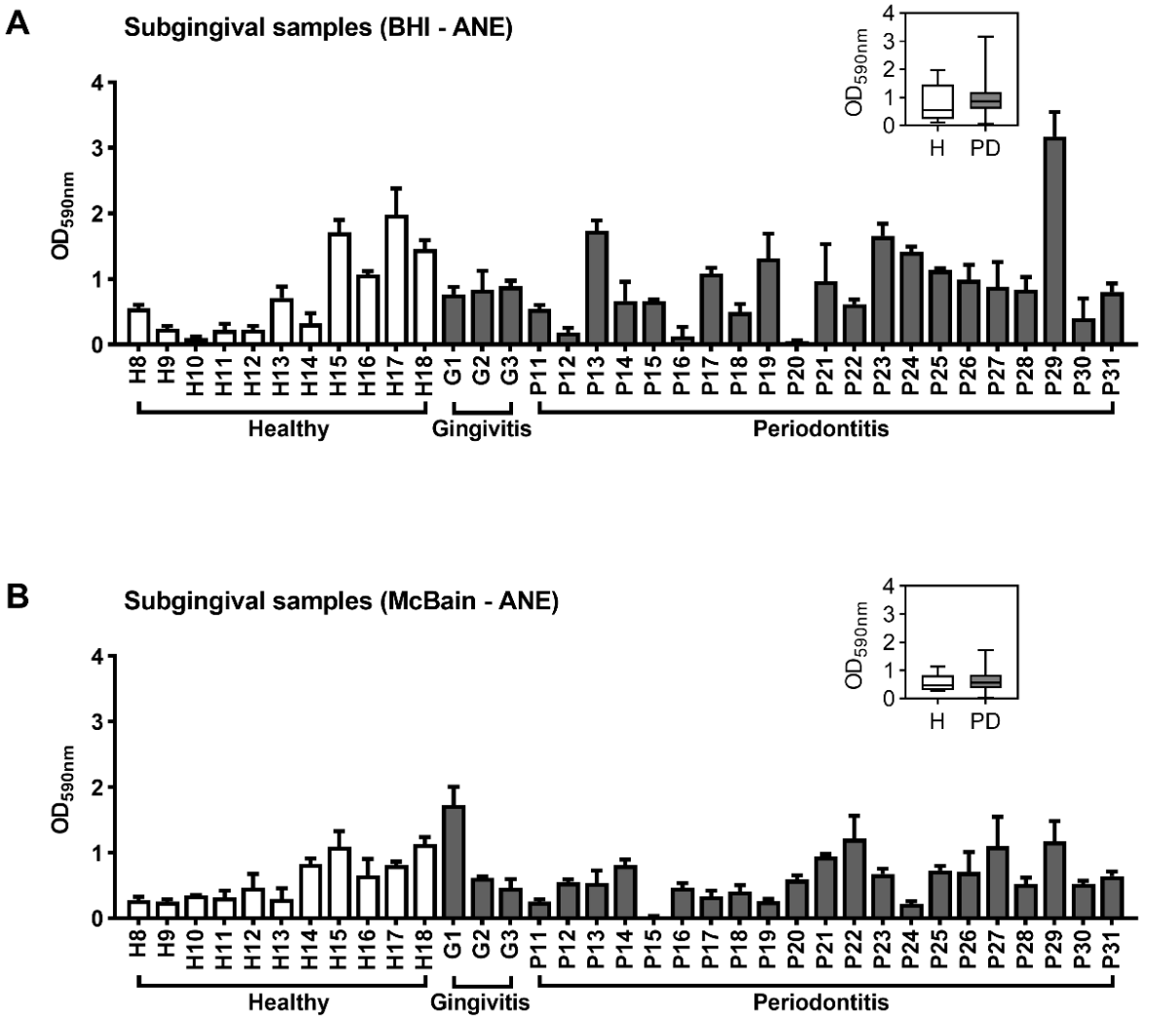


Figure 12. Biofilm formation of subgingival samples in the AAA model. Biofilm quantification is represented as mean ± SD (*n* = 3) of control biofilms (OD_{590nm}). Samples used to inoculate the biofilms were obtained from healthy donors (white bars) and patients with gingivitis and periodontitis (grey bars). All biofilms were grown for 24 hours. Biofilms were cultured in anaerobic conditions in BHI (A) and McBain medium (B). Inserted in each graph is a boxplot (median and interquartile range -IQR-, whiskers range from minimum to maximum) comparing absolute values of control biofilms (OD_{590nm}) from healthy donors (*n* = 11) and patients with gingivitis and periodontitis (*n* = 24). Asterisks (*) indicate statistical significance (*t*-tests, *α = 0.05). ANE: anaerobiosis. Reproduced with permission from Parga et al. (submitted).

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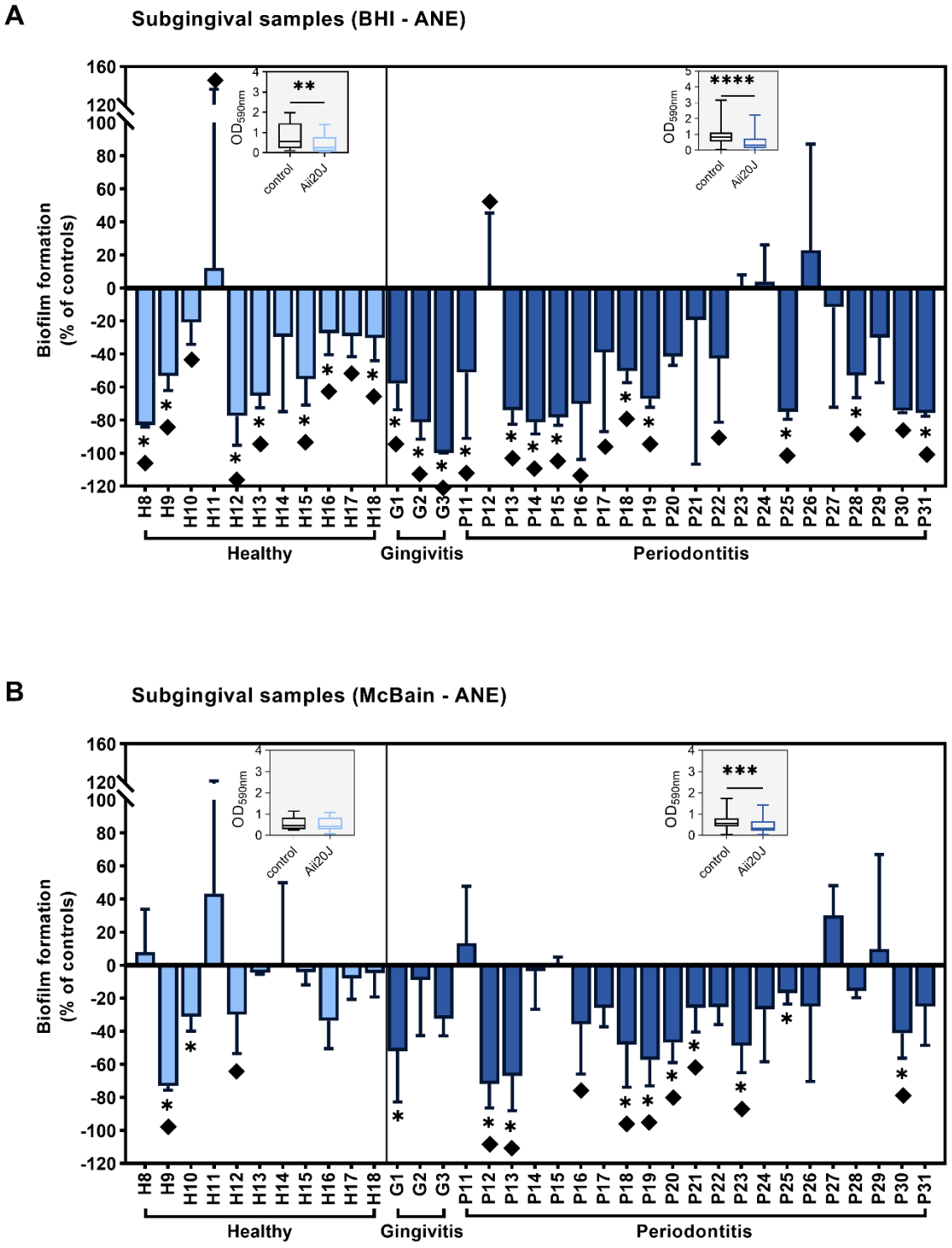


Figure 13. Inhibition of subgingival-derived biofilm formation in the presence of Aii20J. Results are represented as mean ± SD (*n* = 3) of the percentage of biomass in Aii20J-treated biofilms relative to biomass in untreated controls (OD_{590nm}). Samples used to inoculate the biofilms were obtained from healthy donors (light blue bars) and patients with gingivitis or periodontitis (dark blue bars). All biofilms were grown on the AAA model for 24 hours. Subgingival samples were cultured in anaerobiosis in BHI (A) and McBain medium (B). Asterisks (*) indicate statistical significance (*t*-tests, *α = 0.05). Diamonds (♦) represent macroscopical

differences between untreated and Aii20J-treated biofilms. Inserted in each graph is a boxplot (median and interquartile range -IQR-, whiskers range from minimum to maximum) comparing absolute values of biofilm mass (OD_{590nm}) in control and Aii20J-treated biofilms from healthy donors (*n* = 11) and patients with gingivitis and periodontitis (*n* = 24). Asterisks (*) indicate statistical significance (Wilcoxon tests, **α = 0.01, ***α = 0.001, ****α = 0.0001). ANE: anaerobiosis. Reproduced with permission from Parga et al. (submitted).

Biomass reductions in response to Aii20J ranged from 50 – 99% compared with the biomass achieved in control conditions in several samples, and between 20 – 50% in most samples, whereas a small proportion presented little or no biomass reductions when treated with Aii20J. This contrasting behaviour among biofilms generated from different patients has already been described with the antibiotic amoxicillin (Mira et al., 2019). These observations strongly support the use of non-pooled oral samples as inocula to generate *in vitro* biofilms, as it allows for observing different responses among individuals. The assessment of individual responses is of special importance in the current context of oral health care, with a growing consensus towards personalised therapies and several proposals to study individual responses on complex samples before treatment administration (Bartold, 2017; Belibasakis et al., 2019).

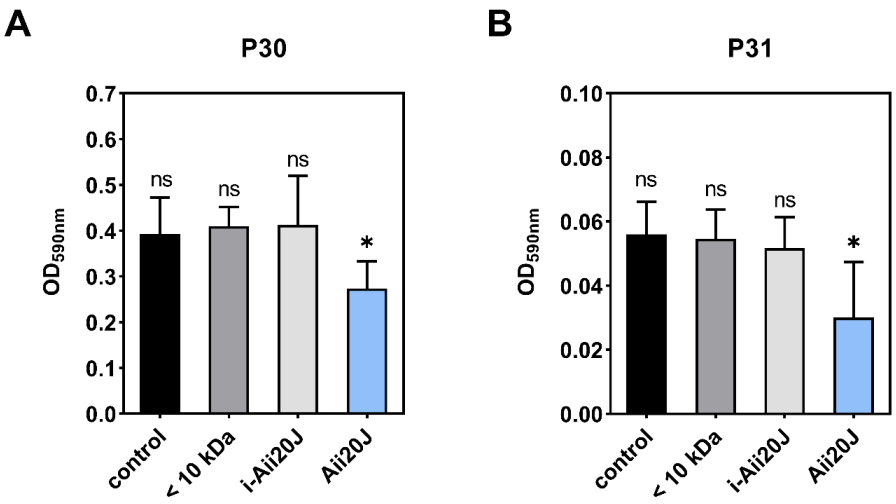


Figure 14. Effect of the QQ enzyme ("Aii20J"), the soluble fraction of the purification product comprising all compounds under 10 kDa ("< 10 kDa"), and the heat-inactivated QQ enzyme ("i-Aii20J") on *in vitro* subgingival biofilm formation in comparison with the untreated biofilms ("control"). Biofilm mass was measured with CV staining (OD_{590nm}). Subgingival samples were grown in McBain and anaerobiosis in the AAA model for 24 hours. Samples used to inoculate the biofilms were obtained from two patients with periodontal disease: P30 (A), and P31 (B), from previous experiments. Results displayed in this figure were generated from independent experiments than the ones included in Figure 13. Results are represented as mean ± SD (*n* = 3) of biofilms. Asterisks (*) indicate statistical significance (ANOVA, *α = 0.05). Reproduced with permission from Parga et al. (submitted).

Subgingival biofilms from patients P30 and P31 grown in McBain were visualised under confocal microscopy to compare the structure of biofilms that presented different responses to Aii20J as assessed with the CV assay (Figure 13B). Biofilms from patient P30 were significantly reduced in biomass in the CV assay and also showed a significant reduction in biofilm coverage when comparing confocal photographs of control (11.0 ± 4.9%) and Aii20J-treated biofilms (5.8 ± 3.7%) (*t*-test) (Figure 15). On the other hand, biofilms from patient P31 presented no differences in biofilm mass in the CV assay nor biofilm coverage between control (3.26 ± 2.57%) and Aii20J-treated biofilms (3.57 ± 1.43%) (*t*-test) (Figure 15). However, we observed that untreated control biofilms were predominantly stained with PI, i.e., contained mostly membrane-damaged cells, whereas Aii20J-treated biofilms were predominantly stained with SYTO 9, indicating that most cells were intact (Figure 15). Thus, even in biofilms that are not reduced in terms of biomass in the presence of Aii20J, the enzyme may affect the biofilm maturation stage.

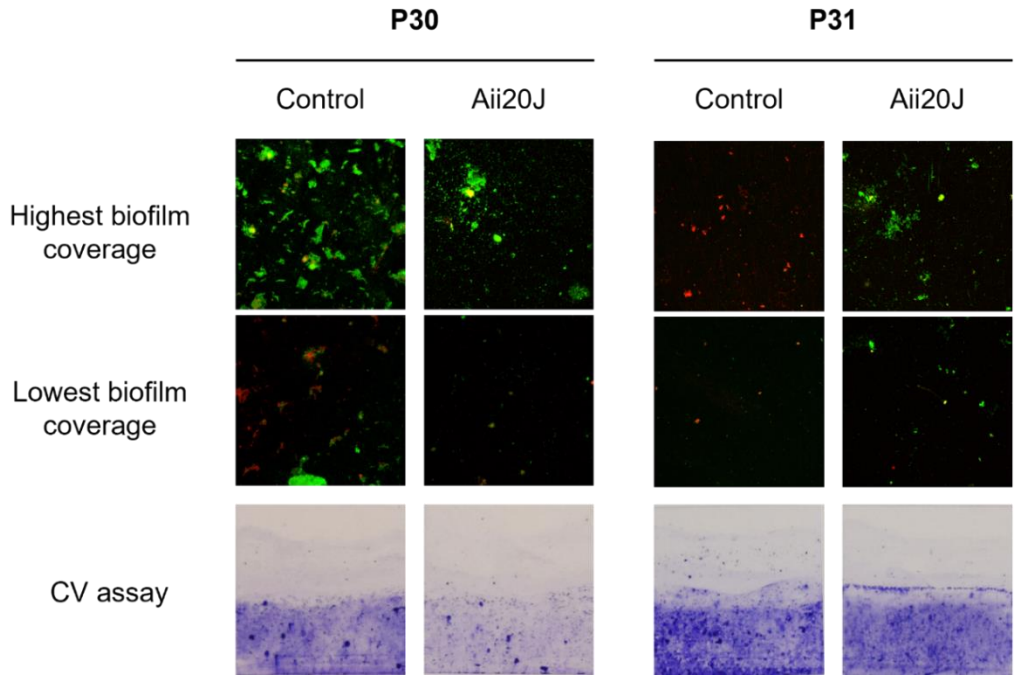


Figure 15. Confocal-laser scanning microscopy photomicrographs of subgingival biofilms grown in the AAA model in McBain and anaerobiosis, in control and Aii20J-treated conditions. Confocal images are compared with CV staining results in the same conditions. Samples were taken from two patients with periodontitis (P30 and P31). Confocal images (20x magnification) show membrane-intact cells in green (stained with SYTO 9) and membrane-damaged cells in red (stained with PI). The figure displays the field with the highest and lowest biofilm coverage for each patient and condition among eight randomly analysed fields. For CV-stained biofilms, one glass coverslip is displayed for each condition and patient. Reproduced with permission from Parga et al. (submitted).

In parallel to biofilm quantification, biofilm mass from control and Aii20J-treated samples grown in McBain was collected for DNA extraction, as we previously observed that this culture medium allowed higher microbial diversity in biofilms from subgingival samples (see Figure 11). Sequencing of the 16S rRNA gene confirmed the dominance of the genus *Streptococcus,* reaching a 45 – 61% relative abundance in the biofilms (Figure 16A,B,C). *Veillonella, Haemophilus, Granulicatella, Gemella,* and *Aggregatibacter* were also found in all biofilms within the top ten most relatively abundant genera. The genus *Streptococcus* does not produce AHLs, and it has already been described that the enzyme Aii20J has no effect on *in vitro* growth or biofilm formation of *S. mutans* (Muras et al., 2018). Hence, the observed biofilm reductions in these streptococcal-dominated communities could be derived from the degradation of AHLs produced by a minority of gram-negatives within the biofilm, or by the degradation of AHL-related molecules.

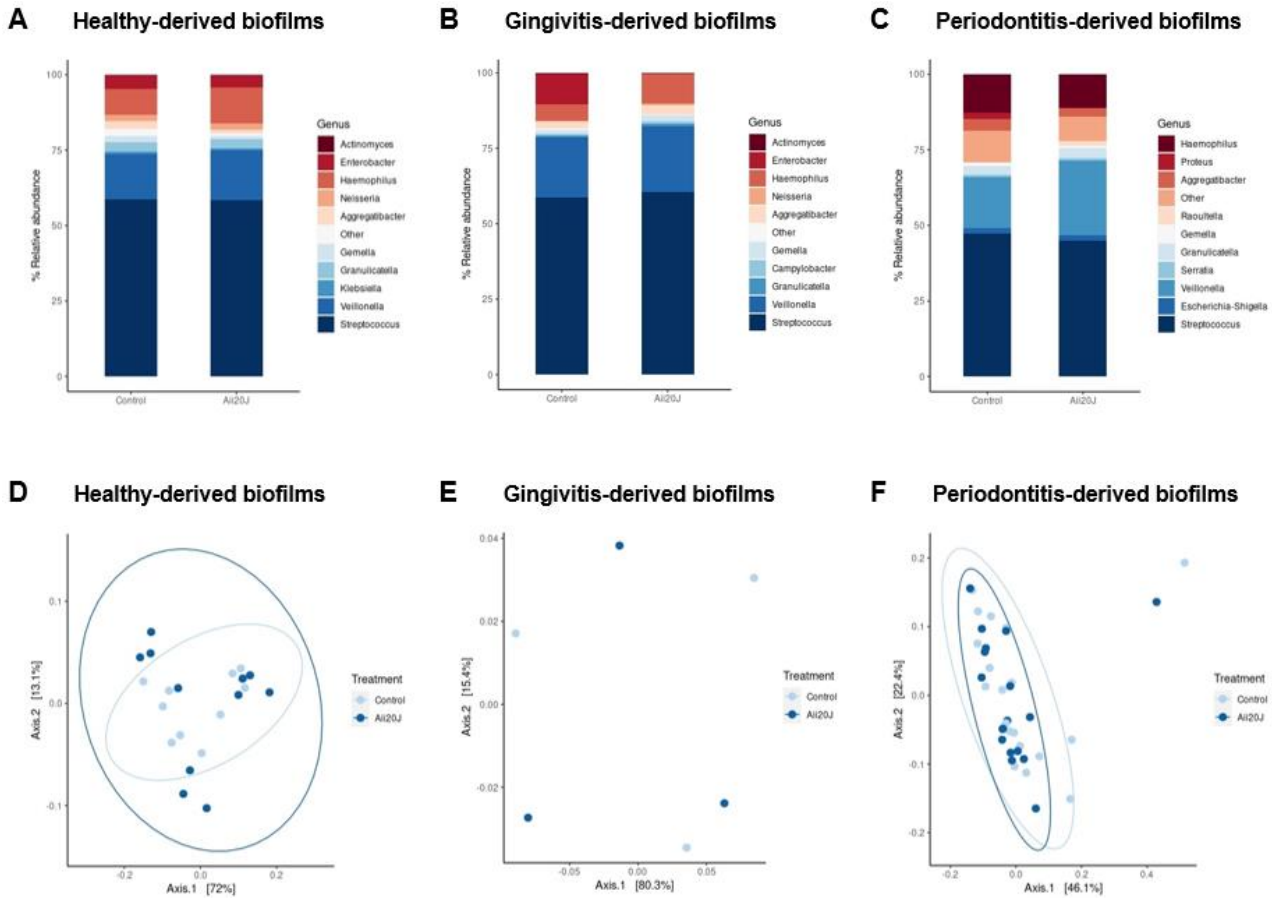


Figure 16. Relative abundance of the bacterial genera identified in the biofilms in the presence of Aii20J. Relative abundance is represented as the percentage of the ten most relatively abundant bacterial genera identified in each biofilm. Genera detected in lower relative abundance appear under the category "Other".

Subgingival samples were grown in the AAA model for 24 hours in McBain, with and without Aii20J. Samples were obtained from (A) healthy donors (*n* = 11), (B) patients with gingivitis (*n* = 3), and (C) patients with periodontitis (*n* = 19). Principal Coordinate Analysis (PCoA) of weighted Unifrac plots of the microbiome structure of the samples obtained from (D) healthy donors (*n* = 11), (E) patients with gingivitis (*n* = 3), and (F) patients with periodontitis (*n* = 19) are shown. Reproduced with permission from Parga et al. (submitted).

Beta diversity examined with Principal Coordinate Analysis (PCoA) (Figure 16D,E,F) and alpha diversity indexes (Figure 17) revealed no significant differences in microbial composition between control and Aii20J-treated biofilms. Since interfering with QS is not expected to directly affect cell viability, major changes in microbiome composition are not expected neither. In fact, similar studies on the effect of lactonases on polymicrobial biofilm communities also reported that the presence of QQ compounds did not change relative abundances in the microbial population (Jo et al., 2016). Conversely, some works did observe changes in the relative abundance of water-associated communities in the presence of lactonases (Huang et al., 2019; Schwab et al., 2019).

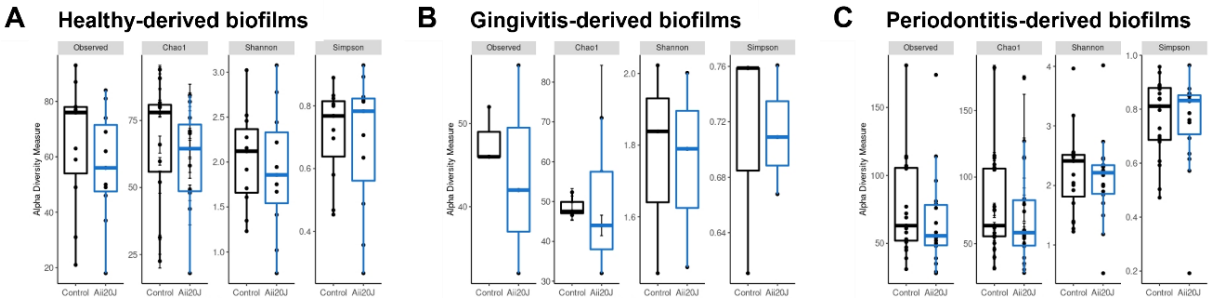


Figure 17. Alpha diversity indexes of subgingival biofilms grown in the AAA model. Boxplots represent the median and interquartile range (IQR), and whiskers range from Q1 - 1.5 IQR to Q3 + 1.5 IQR. Biofilms were grown for 24 hours in McBain and anaerobic conditions. Samples used to inoculate the biofilms were obtained from (A) healthy donors (*n* = 11), (B) patients with gingivitis (*n* = 3), and (C) patients with periodontitis (*n* = 19). Reproduced with permission from Parga et al. (submitted).

A differential abundance analysis was performed, grouping the biofilms according to the oral health status of the donors. In periodontitis-derived biofilms, the genus *Porphyromonas*, among others, significantly decreased its differential abundance in the presence of Aii20J (Figure 18A). Further quantification of *Porphyromonas gingivalis* and *Porphyromonas endodontalis*, both species linked to oral diseases (Hajishengallis et al., 2012; de Brito et al., 2020), was done using qPCR on periodontitis-derived biofilms (*n* = 19). No significant differences were found for *P. gingivalis* nor *P. endodontalis* CFUs between control

and Aii20J-treated biofilms (Mann-Whitney test) (Figure 18B). Total 16S rRNA gene quantification for Eubacteria was performed as a normalisation control.

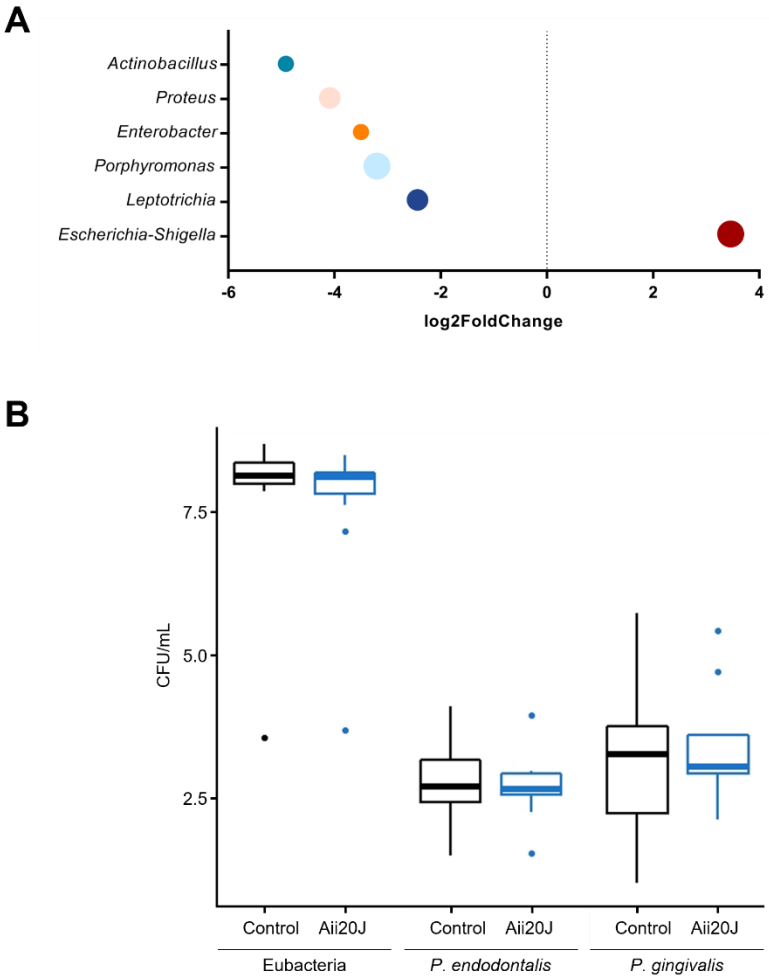


Figure 18. Differential microbial abundance of important genera identified in subgingival, periodontitis-derived biofilms (*n* = 19) in the presence of Aii20J. Samples were grown in the AAA model for 24 hours in McBain, with and without Aii20J. A. Genera with significant (α = 0.05) differential abundance in Aii20J-treated biofilms are shown. Data were analysed using the DESeq2 package (Love et al., 2014). Genera with log2 fold-change values lower than 2 or higher than 2 were considered to be significantly reduced or increased in Aii20J-treated biofilms, respectively. B. Quantification by qPCR of total 16S rRNA from Eubacteria, of *P. gingivalis* and *P. endodontalis*. Untreated control samples are represented by black boxes, and Aii20J-treated biofilms, by blue boxes. Boxplots represent the median and interquartile range (IQR), and whiskers range from Q1 - 1.5 IQR to Q3 + 1.5 IQR. Mann-Whitney tests (α = 0.05) for independent samples were performed. CFU: colony-forming unit. Reproduced with permission from Parga et al. (submitted).

In addition, several studies indicate that the progression of oral diseases, such as periodontitis, is not caused by alterations in the abundance of a specific pathogen but by the

combination of metabolic and functional changes in the entire oral community (Duran-Pinedo and Frias-Lopez, 2015; Yost et al., 2015; Solbiati and Frias-Lopez, 2018). In this sense, although 16S rRNA sequencing studies have provided essential information related to bacterial communities in the oral cavity, we must consider that this methodology provides only taxonomic data but not metabolic or functional information, as we report significant alterations only in phenotypical behaviours.

Last, supernatants of untreated biofilms (n = 16) were investigated for the presence of the most common AHLs (from C_4- to C_{18}-HSL, with or without oxo and hydroxy substituents) by HPLC-MS analysis. Surprisingly, and despite the clear inhibitory effect observed in the presence of the Aii20J lactonase, the production of the most common AHLs was not detected in untreated biofilm cultures, as the AHL that appeared on the biofilms, C_4-HSL, was also detected in fresh McBain culture medium samples included as a control in the HPLC-MS analysis (Figure 19A,B). AHLs such as C_8-HSL have been detected *ex vivo* in extracted teeth, and C_8-HSL, C_{14}-HSL, and C_{18}-HSL have been found *in vivo* in saliva samples (Muras et al., 2020b). In addition, several oral isolates have been demonstrated to produce AHLs in pure or controlled mixed cultures *in vitro* (Kumari et al., 2006; Yin et al., 2012b, 2012a; Chen et al., 2013; Goh et al., 2014, 2016; Muras et al., 2020b), and predicted AHL-related genes had been described in oral genomes (Aleti et al., 2019). Culture conditions in the present study could be affecting the production of AHLs in polymicrobial biofilms *in vitro*, either as a consequence of the selection of non-AHL producer bacteria or as a result of culture conditions affecting AHL producers. The latter has been previously demonstrated in the human pathogen *Acinetobacter*, which switches on and off its AHL-producing routes depending on incubation conditions *in vitro* (Mayer et al., 2018). Moreover, the low sample volume available for extraction could have hampered the detection by HPLC-MS of AHLs at low concentrations. Hence, we cannot fully rule out the presence of AHLs acting at a micro-scale within the biofilms. Despite not being able to confirm the production of AHLs in these *in vitro* biofilms, the presence of AHLs in the culture medium implies an exogenous addition of AHLs to the biofilms and thus provide the Aii20J-treated biofilms with a substrate that the enzyme can degrade.

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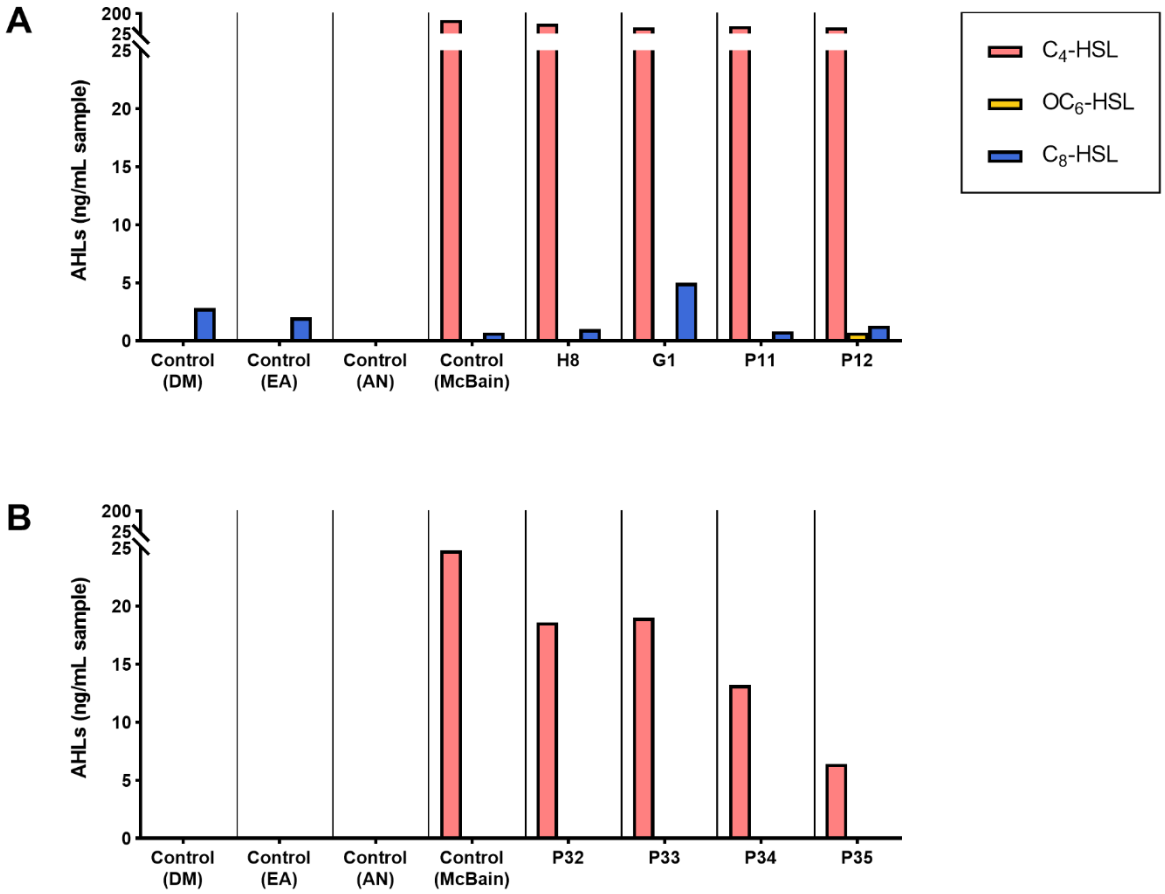


Figure 19. AHL titre in untreated *in vitro* biofilm samples. Biofilms were grown in McBain in anaerobiosis for 24 hours in the AAA model. Negative controls comprised the solvents used for the extraction process: dichloromethane (DM), ethyl acetate (EA), and acetonitrile (AN), and the fresh culture medium used for biofilm generation experiments. Samples were analysed by HPLC-MS, and AHL titre is expressed as nanograms of AHL per millilitre of sample extracted (y-axis). Panels A and B represent two independent experiments, with corresponding controls.

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4. Abundance of QS- and QQ-related sequences in oral metagenomes

The absence of standard AHL production detected in *in vitro* biofilms and the impossibility of performing the same analytical procedures to detect AHLs on the original saliva and subgingival samples due to their reduced volume prompted the investigation of the presence of AHL-related sequences in 125 metagenomes from supragingival plaque samples available in public databases (Belda-Ferre et al., 2012; Human Microbiome Project Consortium, 2012a, 2012b). The metagenomes were investigated using as query a selection of sequences related to AHL synthesis and sensing, and AHL enzymatic degradation. This selection was made by manually searching NCBI's databases (https://www.ncbi.nlm.nih.gov/) for annotated nucleotide and amino acid sequences with demonstrated QS or QQ activity. Selected QS sequences comprised AHL synthases (n = 36), AHL receptors (n = 27), and the AI-2 synthase LuxS (n = 11). Selected QQ sequences included three types of enzymes: lactonases (n = 63), acylases (n = 20), and paraoxonases (n = 3).

The analysis of the supragingival metagenomes revealed a surprisingly high abundance of proteins homologous to the AHL synthase HdtS (Figure 20, Supplementary Table S1; Supplementary_Table_S1.pdf). This protein family comprises three HdtS sequences from *Pseudomonas*, and it appeared in almost every metagenome, almost as abundantly as the four protein families of LuxS included in this analysis (Figure 20). The abundance of LuxS was used as a reference, as it is implicated in central metabolism and, therefore, widely represented in gram-positive and gram-negative bacteria. However, AI-2, the molecule resulting from the detoxification of S-adenosylmethionine, can also act as a QS signal in some species (Schauder et al., 2001; Winzer et al., 2002a). Given the high prevalence of LuxS proteins, as shown in Figure 20, the fact that the response regulator of AI-2, the LuxR protein of *Vibrio harveyi* (Showalter et al., 1990), is absent in the supragingival plaque metagenomes indicates a primary metabolic role of LuxS in these samples. Therefore, the fact that the abundance of the AHL synthase HdtS in these metagenomes is similar to that observed for LuxS suggests an important role of these synthases in the oral environment. HdtS does not belong to the main AHL synthase families, and it has been demonstrated to produce several AHLs, including $OHC_{14:1}$-HSL, an uncommon AHL that can act as a bacteriocin (Laue et al., 2000).

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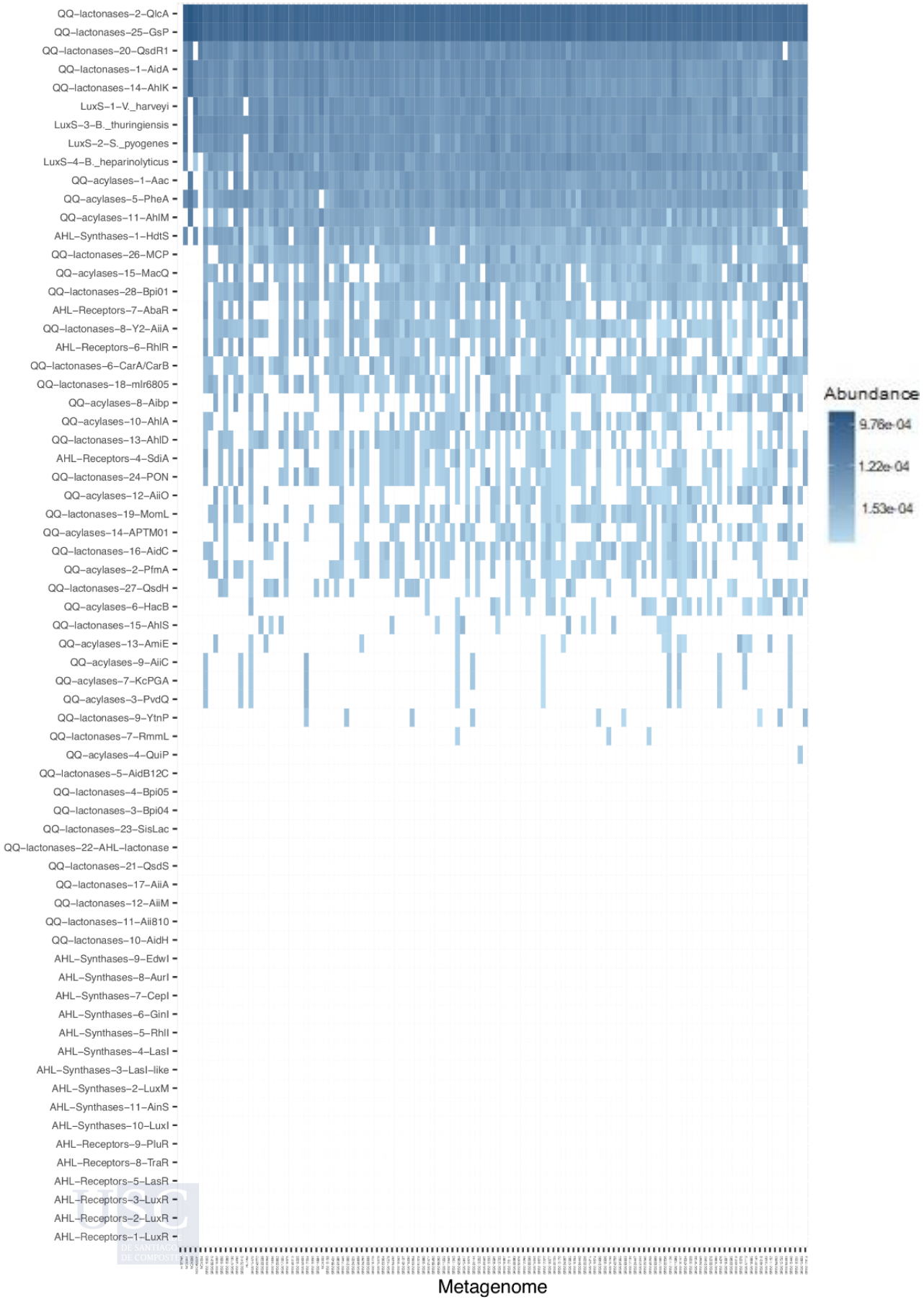

QQ–lactonases–2–QlcA
QQ–lactonases–25–GsP
QQ–lactonases–20–QsdR1
QQ–lactonases–1–AidA
QQ–lactonases–14–AhlK
LuxS–1–V._harveyi
LuxS–3–B._thuringiensis
LuxS–2–S._pyogenes
LuxS–4–B._heparinolyticus
QQ–acylases–1–Aac
QQ–acylases–5–PheA
QQ–acylases–11–AhlM
AHL–Synthases–1–HdtS
QQ–lactonases–26–MCP
QQ–acylases–15–MacQ
QQ–lactonases–28–Bpi01
AHL–Receptors–7–AbaR
QQ–lactonases–8–Y2–AiiA
AHL–Receptors–6–RhlR
QQ–lactonases–6–CarA/CarB
QQ–lactonases–18–mlr6805
QQ–acylases–8–Aibp
QQ–acylases–10–AhlA
QQ–lactonases–13–AhlD
AHL–Receptors–4–SdiA
QQ–lactonases–24–PON
QQ–acylases–12–AiiO
QQ–lactonases–19–MomL
QQ–acylases–14–APTM01
QQ–lactonases–16–AidC
QQ–acylases–2–PfmA
QQ–lactonases–27–QsdH
QQ–acylases–6–HacB
QQ–lactonases–15–AhlS
QQ–acylases–13–AmiE
QQ–acylases–9–AiiC
QQ–acylases–7–KcPGA
QQ–acylases–3–PvdQ
QQ–lactonases–9–YtnP
QQ–lactonases–7–RmmL
QQ–acylases–4–QuiP
QQ–lactonases–5–AidB12C
QQ–lactonases–4–Bpi05
QQ–lactonases–3–Bpi04
QQ–lactonases–23–SisLac
QQ–lactonases–22–AHL–lactonase
QQ–lactonases–21–QsdS
QQ–lactonases–17–AiiA
QQ–lactonases–12–AiiM
QQ–lactonases–11–Aii810
QQ–lactonases–10–AidH
AHL–Synthases–9–EdwI
AHL–Synthases–8–AurI
AHL–Synthases–7–CepI
AHL–Synthases–6–GinI
AHL–Synthases–5–RhlI
AHL–Synthases–4–LasI
AHL–Synthases–3–LasI–like
AHL–Synthases–2–LuxM
AHL–Synthases–11–AinS
AHL–Synthases–10–LuxI
AHL–Receptors–9–PluR
AHL–Receptors–8–TraR
AHL–Receptors–5–LasR
AHL–Receptors–3–LuxR
AHL–Receptors–2–LuxR
AHL–Receptors–1–LuxR
Abundance
9.76e-04
1.22e-04
1.53e-04
Metagenome

Figure 20. Abundance of selected QS and QQ protein families in supragingival shotgun metagenomes. Results are represented as a heatmap where rows are sorted by abundance and columns by non-metric multidimensional scaling distances. The complete set of queried metagenomes is available in Supplementary Table S2 (Supplementary_Table_S2.pdf). Non-detected proteins are represented by blank spaces. Modified from Parga et al. (submitted).

Among the AHL receptors investigated, three protein families appeared in elevated abundance in the supragingival plaque metagenomes. Representative members of these families are AbaR of *Acinetobacter,* RhlR of *Pseudomonas,* and the promiscuous AHL-receptor SdiA of *Burkholderia* (Figure 20). These protein families appeared present in a high number of metagenomes, although their abundance was lower than that observed for HdtS. It is worth mentioning that the protein family represented by RhlR also comprised several SdiA sequences, including those from *Escherichia coli* and *Salmonella,* that were divergent from those classified under the SdiA of the *Burkholderia* family. SdiA is described as a LuxR solo, i.e., a bacterial receptor that cannot produce AHLs but respond to them (Michael et al., 2001). SdiA has been demonstrated to be highly promiscuous, presenting a broad ligand-binding specificity and being able to respond to molecules other than AHLs (Nguyen et al., 2015; Styles and Blackwell, 2018). Hence, we cannot fully disregard the possibility of signal molecules different from AHLs being the cognate ligands of SdiA receptors and present in the oral environment. The increasing volume of studies on LuxR solos has revealed that these receptors can bind a wide range of AHL derivatives and molecules containing aromatic groups derived from fatty acid metabolic pathways (Tobias et al., 2020). Altogether, these results subscribe to those of Aleti et al. (2019), who found putative AHL-biosynthesis genes in the genomes of human oral taxa. suggest a role of uncommon AHLs or AHL-like molecules in oral biofilms that are not identified when using standard AHLs as reference, but that broad-range specificity receptors, such as SdiA and other LuxR solos, could sense.

In addition to AHL synthases and receptors, we investigated the supragingival plaque metagenomes for the presence of AHL-inactivating enzymes: lactonases, acylases, and paraxonases. Protein families of lactonase and acylase enzymes appeared in extremely high abundances, in some cases, even higher than LuxS sequences (Figure 20). Interestingly, the family that comprises Aii20J, the enzyme used throughout this study for antibiofilm purposes, also appeared in several metagenomes, although in low abundance (QQ-lactonases-19-MomL) (Figure 20). These findings align with those obtained *in vitro* on the high prevalence of QQ activity in saliva samples (see Figure 10). The protein family QQ-lactonases-14-AhlK appeared

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among the most abundant groups (Figure 20). This is particularly interesting, as this protein family contains, among others, the enzyme AiiB from *Agrobacterium tumefaciens*, a lactonase with a broad specificity of substrates (Liu et al., 2007). The existence of several QQ enzymes capable of degrading AHL derivatives and even molecules other than AHLs has been described in the literature (Hiblot et al., 2013; Bergonzi et al., 2019). The high prevalence in supragingival plaque metagenomes of QQ enzymes that may be degrading signals other than AHLs aligns with the finding of promiscuous receptors in those same metagenomes (Figure 20), strengthening the idea of the presence of AHL-like molecules in the oral cavity. Therefore, we hypothesise that the biofilm inhibitory effect described in this work with the use of Aii20J may not be derived from its action on AHLs but on other signal molecules of related structure. A similar case has already been described with the QQ lactonase SsoPox. The antibiofilm activity of this enzyme against *Pseudomonas aeruginosa* monospecies biofilm is more dependent on its promiscuous activity on the *P. aeruginosa* PQS signal than on its activity against AHLs (Rémy et al., 2020). When Rémy et al. (2020) compared SsoPox with a lactonase more active against AHLs, but without activity against PQS, the latter presented a much lower antibiofilm activity, suggesting that lactonases with wider substrate ranges have greater antibiofilm properties. Hence, our results on the antibiofilm activity of Aii20J on oral samples could be explained by a possible susceptibility of uncommon AHLs or AHL-like molecules to the enzymatic activity of Aii20J. These yet-to-be-identified signals would evidence a much more complex signalling network in oral biofilms than currently established. Furthermore, the effect of Aii20J on biofilms dominated by *Streptococcus*, a gram-positive that is not expected to respond to AHLs and is not affected by this QQ enzyme in *in vitro* pure cultures (Muras et al., 2018), could be partially explained by the introduction of non-AHL signals in the scenery. Future investigations on the substrate specificity of Aii20J towards molecules other than standard AHLs will help validate this hypothesis.

Findings in this work suggest an important role of AHLs or AHL-like molecules in oral biofilm formation. Targeting these molecules results in significant biomass reductions *in vitro* with no significant alterations in microbial relative abundance. Furthermore, this study is first in describing a remarkably high abundance of sequences related to AHL synthases and promiscuous AHL receptors on supragingival plaque metagenomes. Overall, these results indicate that QQ strategies could be useful for controlling oral biofilm formation while highlighting the importance of a personalised assessment at the clinical practice.

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This work investigated the potential of the QQ enzyme Aii20J to prevent oral biofilm formation based on *in vitro* studies. Given the rise of antimicrobial resistance in dentistry, alternative approaches to control periodontal diseases need to be explored (Cieplik et al., 2014; Rams et al., 2014). Several studies have described the effectiveness of QQ strategies against monospecific (Sun et al., 2021) and polymicrobial biofilms (Jo et al., 2016; Huang et al., 2019; Schwab et al., 2019; Jeong et al., 2020). Specifically, the inhibitory effect of the AHL-lactonase Aii20J on biofilm formation obtained from saliva samples has already been described (Muras et al., 2020b). Aii20J is a wide-spectrum, extremely thermoresistant enzyme that has no genotoxicity (UNE-EN ISO 10993-3:2015) and no cytotoxicity in eukaryotic cells (OECD Guideline 471) (unpublished results). Besides, we have confirmed that Aii20J retains its activity after 24 months in a commercial mouthwash at room temperature (unpublished results). Despite being a member of the metallo-beta-lactamase super-family, Aii20J does not affect beta-lactam antibiotics (Mayer et al., 2015) and does not interfere with bacterial growth (Muras et al., 2018), reducing the risk of inducing antimicrobial resistance. Altogether, these properties make Aii20J a promising candidate for developing antibiofilm strategies in the oral health context.

Despite these promising results, further *in vitro* studies, including the evaluation of the effect of Aii20J at a transcriptional and proteomic level, are required to understand the mechanisms underneath the behavioural changes detected in these biofilms. Additionally, the investigation of subgingival metagenomes for the presence of AHL-related sequences would shed light on the extent of their distribution within the oral cavity. The final goal of testing QS-targeting strategies would be their transfer from *in vitro* experiments to *in vivo* and clinical trials. To do so, it would be interesting to assess possible synergies of Aii20J with antimicrobials beforehand. Also, it would be necessary to assess the optimal *in vivo* delivery doses, retaining time, and other pharmacological aspects when considering the addition of the QQ enzyme to oral products.

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CHAPTER 2 | Inhibition of caries biofilms *in vitro* using Quorum Quenching

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Background

Caries is an oral disease that affects children and adults worldwide. The most recent estimate points to 520 million children suffering from caries in deciduous teeth and 2000 million people with caries in permanent teeth (Global Burden of Disease Collaborative Network, 2020). Dental caries is a costly disease in terms of treatment, but one of its most important consequences is tooth decay. When left untreated, caries can progress into more severe diseases, such as root canal infections, and eventually lead to bloodstream infections and endocarditis.

Dental caries is a polymicrobial disease which starts with a disturbance in a healthy oral biofilm (Simón-Soro and Mira, 2015). This disturbance is commonly due to an increased intake of fermentable sugars and carbohydrates that are available for bacteria to turn into acidic compounds. A lowered pH maintained through time eventually surpasses the homeostatic mechanisms of the healthy oral biofilm through the selection of acidogenic and aciduric microorganisms, creating a shift towards a cariogenic biofilm (Bradshaw and Lynch, 2013; Sheiham and James, 2015; Boisen et al., 2021). Together with the presence of acidogenic and aciduric bacteria and the availability of fermentable carbohydrates, there is the factor of host susceptibility, defined by the unique combination of genetics and environment of each person (Selwitz et al., 2007; Lif Holgerson et al., 2015). The consensus on the aetiology of caries has been evolving and adapting to newly discovered aspects of the disease. It is now accepted that the functional activities within oral biofilms can sometimes have a greater role in the progression of diseases than the microbiome structure (Takahashi and Nyvad, 2011; Duran-Pinedo and Frias-Lopez, 2015; Simón-Soro and Mira, 2015).

The current battle against caries relies on the prevention of the disease, with approaches ranging from dietary sugar and carbohydrate restrictions and increased oral hygiene, to probiotics and prebiotics (see the Introduction section). The search for novel alternative strategies to treat and prevent oral diseases has pointed out the possibility of interfering with cariogenic virulence factors without affecting bacterial viability (Muras et al., 2018, 2020b; Philip et al., 2018; Muras and Otero, 2020). One of these cariogenic factors is bacterial communication, also known as Quorum Sensing (QS). The presence of the main QS pathways has been described in caries-associated species and species retrieved from carious lesions. The use of autoinducer peptides has been described in several streptococci, where QS is mainly dependent on the competence-stimulating peptide (CSP). Interference with the CSP signalling

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system in *S. mutans* has been shown to inactivate virulence factors and impair the persistence of this bacterium in the oral biofilm (Cvitkovitch et al., 2003; Qi et al., 2005). The universal autoinducer-2 (AI-2) has been described in several oral pathogens, specifically streptococci (Frias et al., 2001; Rickard et al., 2006). Furthermore, the use of a cellular extract from *Tenacibaculum* sp. 20J active against AI-2 was shown to reduce *S. mutans* biofilm formation *in vitro*, possibly by inhibiting AI-2 signalling (Muras et al., 2018). *N*-acyl-homoserine lactones (AHLs), typically used by gram-negative bacteria, had been classically considered non-relevant in the oral cavity (Frias et al., 2001; Jakubovics and Kolenbrander, 2010; Huang et al., 2011b; Guo et al., 2014). Nevertheless, AHLs have been found in saliva and extracted teeth (Kumari et al., 2006; Muras et al., 2020b), and bacterial strains from dentine caries have been shown to produce AHLs (Goh et al., 2014, 2016). Moreover, bacterial gene clusters predicted to be related to homoserine lactone biosynthesis have been described in the saliva metagenomes of children with caries (Aleti et al., 2019). In addition, two studies on *in vitro* oral biofilms exposed to AHLs and modified AHLs reduced lactic acid accumulation in the presence of fermentable sugars without affecting bacterial growth (Janus et al., 2016; Muras et al., 2020a). Still, the extent of the role of AHLs in the oral cavity remains scarcely investigated. Altogether, this evidence points to the possibility of interfering with AHL-mediated communication mechanisms to modulate oral biofilm formation.

In this study, we aimed to characterise the effect of the QQ enzyme Aii20J on *in vitro* biofilms from caries-free children and children with caries. The microbiome structure of the initial supragingival biofilms was investigated by 16S rRNA sequencing, and these supragingival samples were used to generate *in vitro* biofilms in the presence of the enzyme. The effect of Aii20J was investigated regarding biofilm mass (CV assay) and microbial composition (Illumina sequencing of the 16S rRNA gene) of the *in vitro* biofilms. This study describes reductions in biomass of *in vitro* biofilms generated from samples of caries-free children and children with caries upon treatment with Aii20J. These observations were not accompanied by changes in the microbial diversity of the biofilms, as previously described for subgingival samples from adult patients (see Chapter 1).

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Results and discussion

1. Microbial composition of supragingival biofilm samples from caries-free children and children with caries

Supragingival biofilm samples were taken from caries-free children and children with caries (see Table 5 in Methods section). To investigate their microbiome structure, gDNA was extracted to sequence the V3-V4 hypervariable regions of the 16S rRNA gene via Illumina.

Figure 21A,B shows the relative abundance of the most abundant genera identified in supragingival biofilm samples from caries-free children and children with caries. Streptococci were found in relative abundances ranging from 2 – 34% among caries-free and caries patients. Such proportions are similar to those compiled in the Human Oral Microbiome Database (HOMD) based on the studies of Eren et al. (2014) and of Segata et al. (2012), with reported relative abundances of 15 – 30% for the genus *Streptococcus* in supragingival plaque samples. Besides *Streptococcus,* the most relatively abundant genera were *Leptotrichia, Veillonella, Fusobacterium, Capnocytophaga, Prevotella, Neisseria, Corynebacterium, Porphyromonas* and *Selenomonas*. Yet, supragingival biofilms comprised a much higher number of taxa, with 38 – 71 total genera identified among samples (Table 9). The less relatively abundant genera comprised proportions of up to 40% of the supragingival biofilms (Figure 21B). The only exception was sample Ca8, taken from a caries patient, in which the predominant genera were *Lactococcus* and *Serratia*, with relative abundances of 34% and 32%, respectively. Besides, this was the only sample in which these two genera were identified (Figure 21A). In fact, principal components analysis (PCA) of the microbiome structure of the supragingival biofilm samples revealed that patient Ca8 was noticeably separated from the others (Figure 21C). After excluding sample Ca8, the microbiome structure of the supragingival biofilms was analysed again, as shown in Figure 21D. The new PCA displayed an almost complete overlapping between samples from caries-free children (yellow) and children with caries (blue). Thus, within this collection of supragingival biofilm samples, the microbiome structures of caries-free and caries children are not significantly different.

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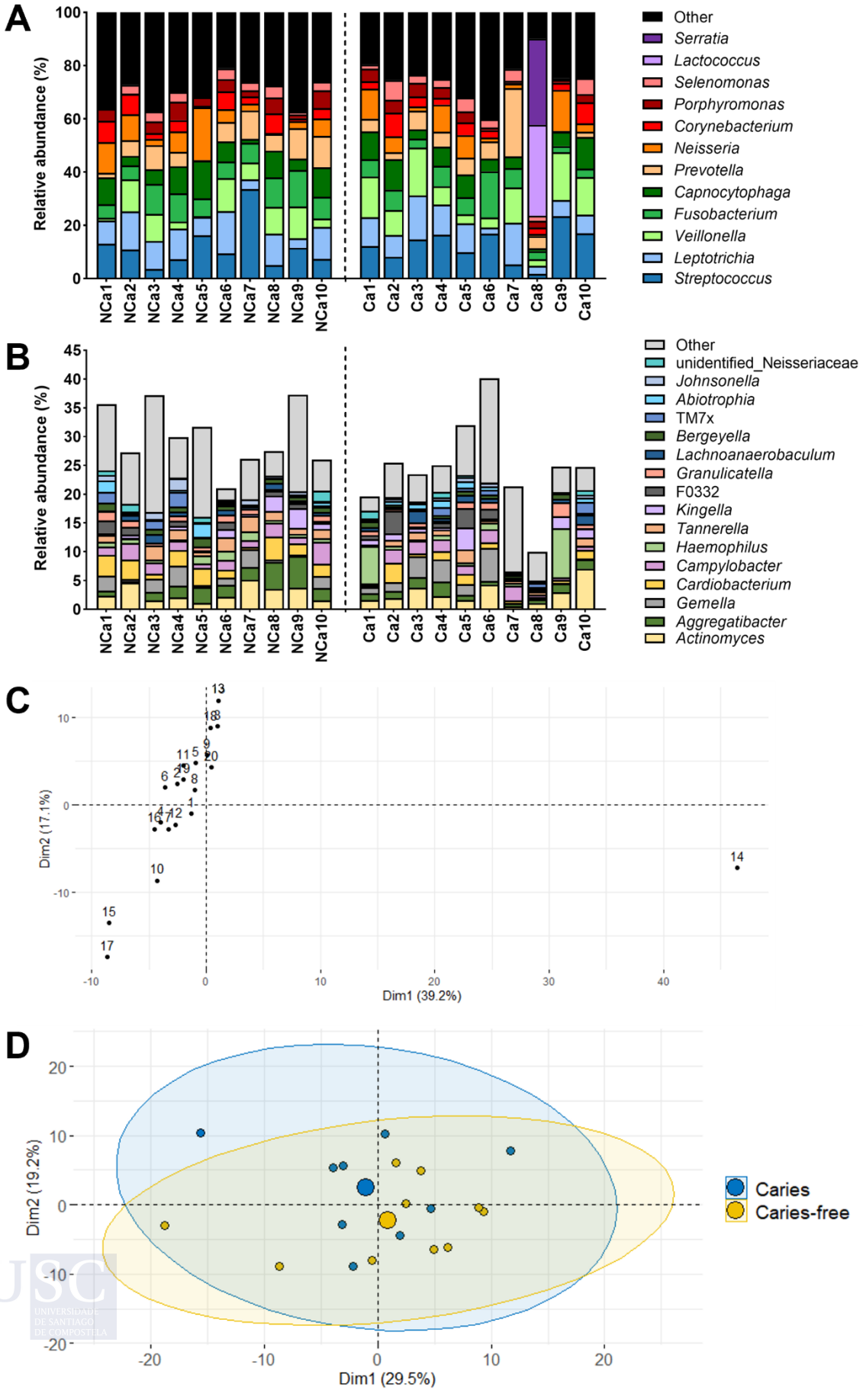

A
Relative abundance (%)
NCa1 NCa2 NCa3 NCa4 NCa5 NCa6 NCa7 NCa8 NCa9 NCa10 Ca1 Ca2 Ca3 Ca4 Ca5 Ca6 Ca7 Ca8 Ca9 Ca10
Other
Serratia
Lactococcus
Selenomonas
Porphyromonas
Corynebacterium
Neisseria
Prevotella
Capnocytophaga
Fusobacterium
Veillonella
Leptotrichia
Streptococcus
B
Relative abundance (%)
Other
unidentified_Neisseriaceae
Johnsonella
Abiotrophia
TM7x
Bergeyella
Lachnoanaerobaculum
Granulicatella
F0332
Kingella
Tannerella
Haemophilus
Campylobacter
Cardiobacterium
Gemella
Aggregatibacter
Actinomyces
C
Dim2 (17.1%)
Dim1 (39.2%)
D
Dim2 (19.2%)
Dim1 (29.5%)
Caries
Caries-free

Figure 21. Relative abundance of the most abundant bacterial genera identified in the supragingival biofilm samples and Principal Components Analysis (PCA) plots of their microbiome structure. Supragingival biofilms were sampled with a curette from caries-free children (*n* = 10) and children with caries (*n* = 10). **A.** Relative abundance is represented as the percentage of the 12 most relatively abundant bacterial genera identified in the samples (y-axis). Genera detected in lower relative abundance appear under the category "Other". Supragingival samples from caries-free children (NCa) are shown on the left part of the x-axis, and samples from children with caries (Ca) are on the right part of the x-axis. **B.** Relative abundance of the genera grouped under the category "Other" that were identified in relative abundance higher than 0.5%. Genera detected in relative abundances lower than 0.5% appear under the category "Other". **C.** PCA plot of the microbiome structure of the 20 supragingival biofilm samples. **D.** PCA plot of the microbiome structure of 19 supragingival biofilm samples (*n* = 10 caries-free children, represented by yellow circles; *n* = 9 children with caries, represented by blue circles), after removing sample "14", corresponding to patient Ca8.

Table 9. Bacterial diversity in supragingival samples of caries-free children (NCa) and children with caries (Ca). Each row represents the number of genera identified in each sample, the number of observed OTUs in each sample, and the Shannon Index (H'), calculated at the OTUs level.

Caries-free patients	**NCa1**	**NCa2**	**NCa3**	**NCa4**	**NCa5**	**NCa6**	**NCa7**	**NCa8**	**NCa9**	**NCa10**
Number of observed genera	53	49	70	55	47	57	50	60	61	50
Number of observed OTUs	106	92	144	108	84	115	98	133	129	99
Shannon Index (H')	3.6	3.3	3.9	3.8	3.2	3.5	3.0	3.7	3.6	3.9
Caries patients	**Ca1**	**Ca2**	**Ca3**	**Ca4**	**Ca5**	**Ca6**	**Ca7**	**Ca8**	**Ca9**	**Ca10**
Number of observed genera	47	52	54	60	60	70	71	65	38	44
Number of observed OTUs	97	104	111	130	131	145	157	129	74	79
Shannon Index (H')	3.3	3.6	3.4	3.6	3.8	3.7	3.7	2.4	2.7	3.1

These observations align with those of Belda-Ferre et al. (2015), who did not find significant differences in the microbiome structure of supragingival plaque samples collected from healthy adults and patients with caries. It is relevant to note here that Belda-Ferre et al. (2015) used supragingival plaque samples taken from intact tooth surfaces, as in the present study, and not directly from the carious lesions. Interestingly, the protein profiles of those subgingival samples were clustered according to the caries status of the subjects (Belda-Ferre et al., 2015). Indeed, in recent years emphasis has been put on the functional activities within

oral microbial communities rather than on their microbiota distribution, as the former has been described to account for a greater impact on disease evolution (Duran-Pinedo and Frias-Lopez, 2015; Simón-Soro and Mira, 2015).

Interestingly, when examining the microbiome of the samples at the genus level with a LDA, genera that appeared at very low relative abundances showed significant differential abundances between caries subjects and caries-free donors (Figure 22). Figure 22 shows positive fold-change values for genera that were more abundant in samples from children with caries than in samples from caries-free children, whereas negative fold-change values represent the opposite.

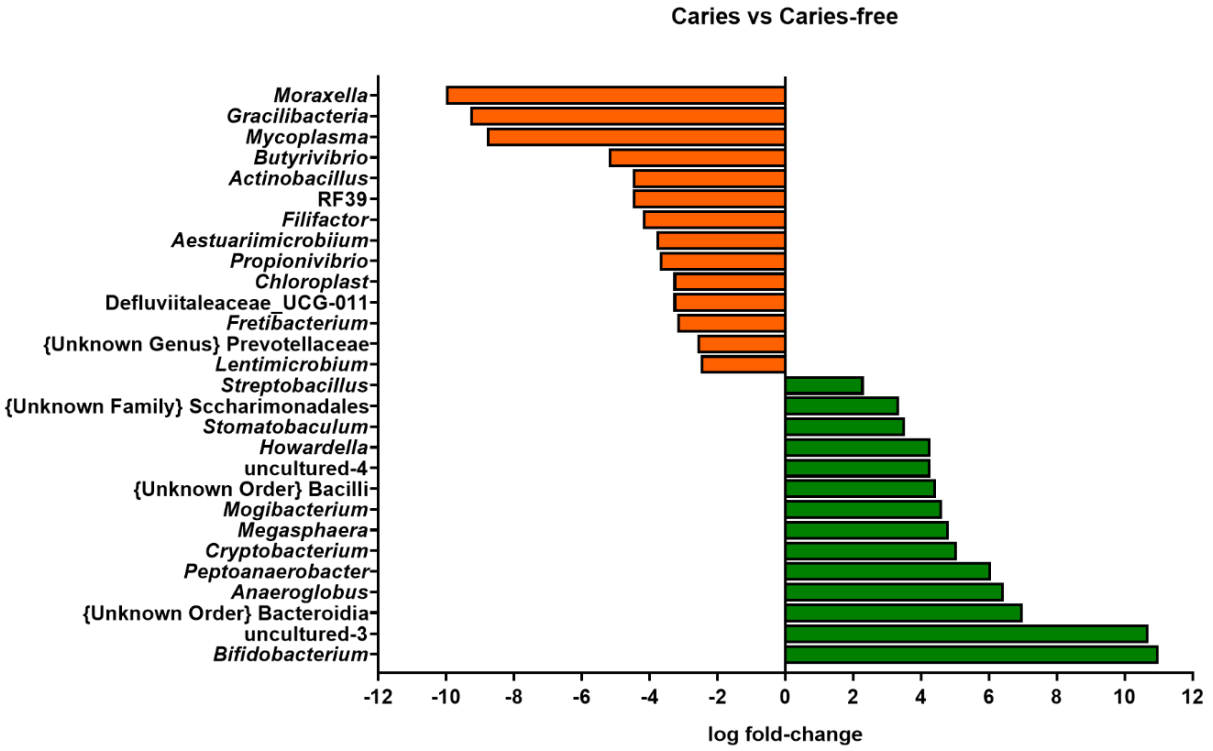


Figure 22. Linear Discriminant Analysis of the genera identified in supragingival biofilm samples (*n* = 20). Supragingival samples were collected from the vestibular and lingual surfaces with a curette. Genera with log fold-change values lower than 2 (orange bars) or higher than 2 (green bars) were considered to be significantly reduced or increased in subjects with caries, respectively.

Whereas the genera classically associated with caries did not present relevant differences in abundance between caries-free and caries subjects, low-abundance genera presented significant differences among caries status. The genus *Bifidobacterium* was increased in caries samples. *Bifidobacterium* members can be highly acidogenic, to the point of initiating dental caries or aiding in their progression (Meurman, 2005; Philip et al., 2018). Nevertheless,

their increased presence in caries subjects could also be derived from a selection of acidogenic bacteria in the acidified cariogenic oral biofilm. On caries-free samples, the genus *Moraxella* appeared with a significantly increased abundance compared with caries samples. This observation aligns with the study of Belda-Ferre et al. (2012), in which they described a tendency of Gammaproteobacteria members to be more common in healthy subjects. The overall homogeneity of the principal genera identified among biofilms of different oral health status subjects reinforces the need for proteomic or metatranscriptomic studies that allow for observing the differences in functional activities among samples and discerning the role of the lower abundance taxa that are differentially represented in caries-free and caries subjects.

2. *In vitro* biofilm formation from caries-free children and children with caries

In vitro biofilms were generated using supragingival biofilm samples as inocula. Supragingival biofilms from caries-free children and children with caries were inoculated in aerobic conditions in the AAA model (Exterkate et al., 2010; Muras et al., 2020a) in McBain medium and, in parallel, in McBain supplemented with 0.2% sucrose. The use of McBain medium aimed to generate diverse biofilms (see Chapter 1). In contrast, the use of McBain in combination with sucrose has already been established as a successful *in vitro* setting for developing cariogenic multispecies biofilms (Janus et al., 2015).

Figure 23 shows the values of total attached aerobic biofilm mass derived from supragingival biofilms grown without Aii20J (control conditions). Biomass values, as assessed with CV assay, were heterogeneous among different patients in both culture media used (Figure 23A,B). Comparison of the samples grouped according to the oral health status of the patients revealed no statistically significant differences between biofilm formation abilities of samples from caries-free children and children with caries in neither of the culture media used ($p = 0,8412$ for biofilms grown in McBain; $p = 0,4143$ for biofilms grown in McBain-sucrose) (Figure 23C,D). These results showed that interindividual differences had a greater influence on *in vitro* biofilm formation abilities than the oral health status of the patient. This observation has been already reported in saliva and subgingival samples (see Chapter 1). Our previous investigation on the microbiome structure of the inocula used to generate these biofilms did not reveal significant differences between patients (Figure 21). Nevertheless, these apparently equivalent microbial populations have different biofilm formation abilities upon *in vitro* cultivation. These results align with the increasing importance given to functional activities

within microbial populations rather than only microbial composition (Duran-Pinedo and Frias-Lopez, 2015; Solbiati and Frias-Lopez, 2018). Biomass quantification of biofilms grown in McBain ranged between 0.1 and 0.4 in absorbance (OD_{590nm}) (Figure 23A), whereas biomass quantification of biofilms grown in McBain supplemented with sucrose ranged between 0.2 and 1.2 (Figure 23B). These results obtained with CV assay subscribe to those of Janus et al. (2015); using the same culture media as in this study, they described that saliva-derived biofilms cultivated in the presence of sucrose achieved higher growth values and rates compared with biofilms cultivated without sucrose.

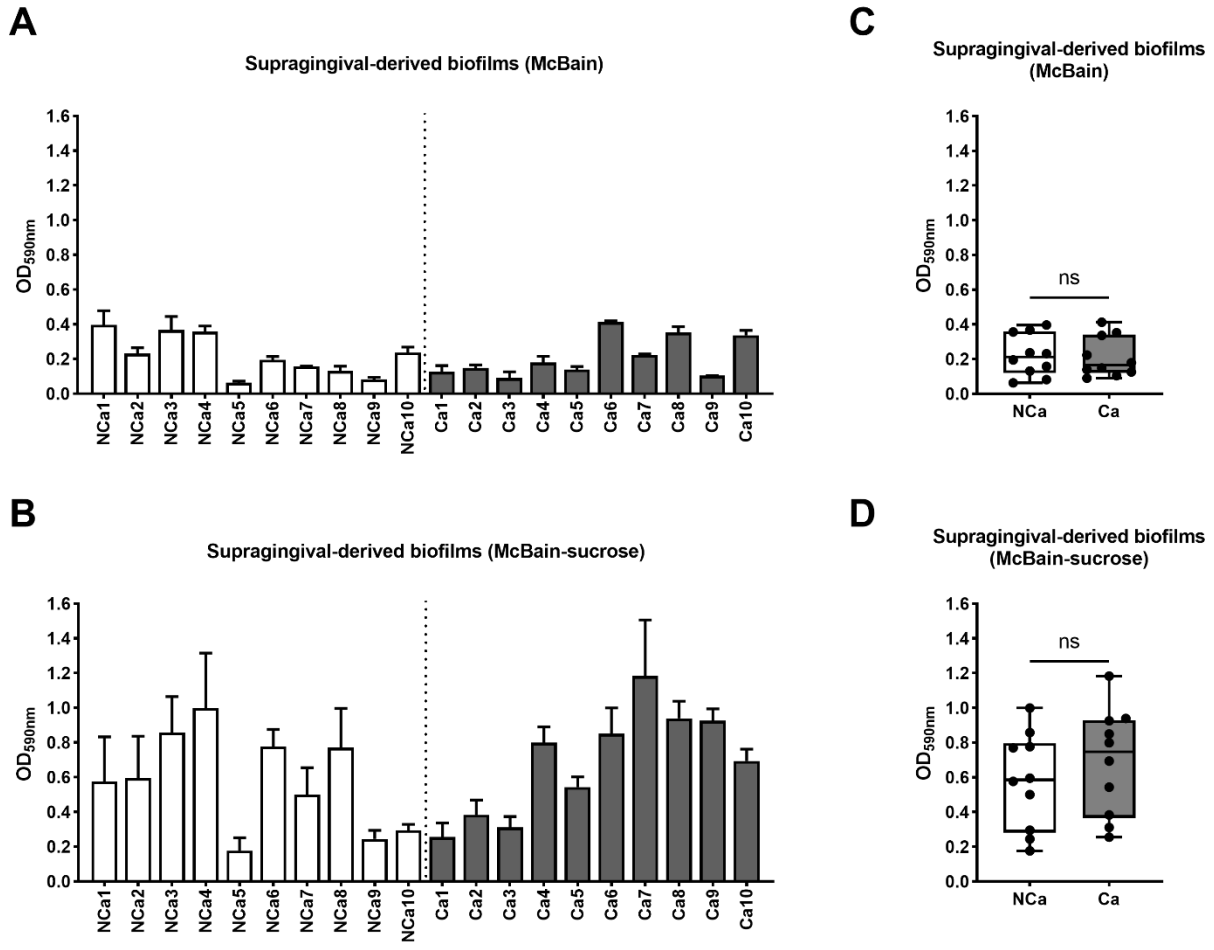


Figure 23. Quantification of *in vitro* supragingival-derived biofilms. Histograms **(A, B)** represent the amount of biofilm formed by each sample without Aii20J treatment (untreated controls), assessed by CV assays (y-axis). Results are expressed as mean of OD_{590nm} measurements, and error bars represent SD (*n* = 3). White bars represent biofilms from samples of caries-free children, whereas grey bars represent biofilms from samples of children with caries (x-axis). Supragingival samples were grown in McBain medium **(A, C)** and McBain medium supplemented with 0.2% sucrose **(B, D).** Boxplots **(B, D)** represent biofilm formation values (y-axis) of all samples from caries-free children (white boxes, *n* = 10) and children with caries (grey boxes, *n* = 10). Boxplots represent the median and interquartile range of these groups of samples, and whiskers range from minimum to maximum. Statistical

significance of comparisons between biofilm formation values in the caries-free and caries group in either culture medium was assessed with unpaired *t*-tests (α = 0.05). ns: not significant.

3. Effect of Aii20J on supragingival-derived biofilms from caries-free children and children with caries

To evaluate the antibiofilm potential of Aii20J, differences in biofilm mass obtained in the presence of this enzyme were compared with that of untreated controls. Figure 24A,B displays values of biofilm quantification after treatment with Aii20J, represented as the percentage of biofilm mass achieved relative to untreated controls, in both culture conditions studied. Overall, biofilm mass was reduced in the presence of the QQ enzyme Aii20J. In biofilms grown in McBain, macroscopical differences were observed in 40% of both caries-free and caries-derived biofilms (Figure 24A). Additionally, statistically significant differences were observed for 20% of both caries-free and caries-derived biofilms (*t*-test) (Figure 24A). In biofilms grown in the presence of sucrose, macroscopical differences were observed in 50% of caries-free- and 60% of caries-derived biofilms, while statistically significant differences were found in 40% of caries-free- and 10% of caries-derived biofilms (Figure 24B).

The effect of Aii20J on the biofilms was further analysed by grouping the samples according to the oral health status of the patient. Figure 25A,B shows "before and after" graphical representations of the results of paired *t*-tests comparing biofilm mass values in untreated biofilms after treatment with Aii20J. These analyses revealed statistically significant differences upon Aii20J-treatment in the caries-free group of biofilms grown in McBain (Figure 25A), and both the caries-free and the caries groups of biofilms grown in McBain with sucrose (Figure 25B). Taken together, these results appear to indicate that biofilms grown in the presence of sucrose respond to Aii20J with greater biomass reductions than those grown without sucrose. The selection of saccharolytic bacteria present in the initial supragingival biofilms is expected with the use of a culture medium supplemented with sucrose. It would appear as if the presence of the enzyme Aii20J had a greater effect on these bacteria than on bacteria selected in the non-sucrose medium.

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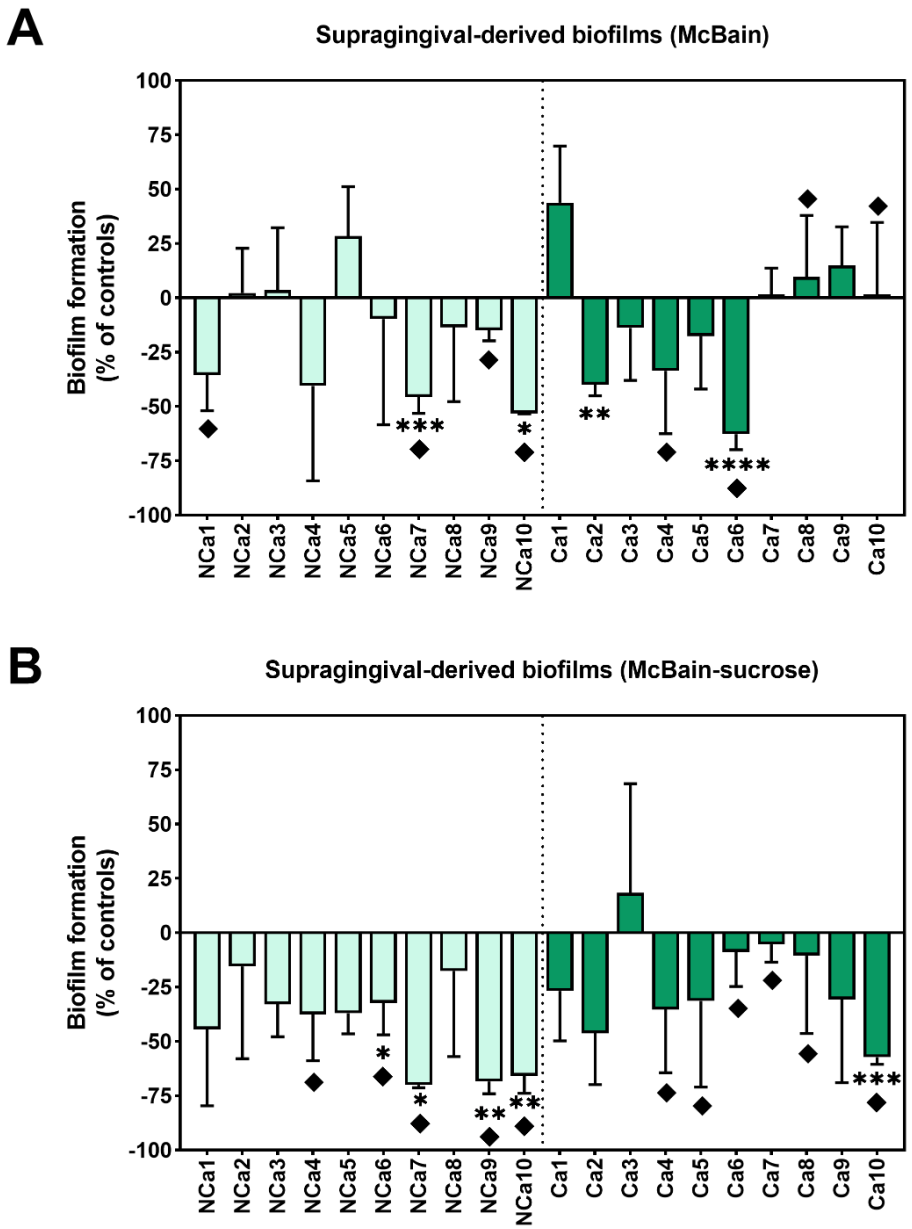


Figure 24. Biofilm formation of supragingival biofilm samples in the presence of Aii20J. Histograms represent the amount of biofilm formed in Aii20J-treated samples as a percentage of the biofilm formed in untreated controls, assessed by CV assay (y-axis). Results are expressed as mean, and error bars represent SD (*n* = 3). Supragingival samples used to inoculate the biofilms were obtained from caries-free children (light green bars) and children with caries (dark green bars). Biofilms were generated in McBain medium **(A),** and McBain supplemented with 0.2% sucrose **(B).** Diamonds (♦) represent macroscopical differences between untreated and Aii20J-treated biofilms. Statistical significance of pairwise comparisons between each Ai20J-treated biofilm and its untreated controls is displayed as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (*t*-tests, α = 0.05).

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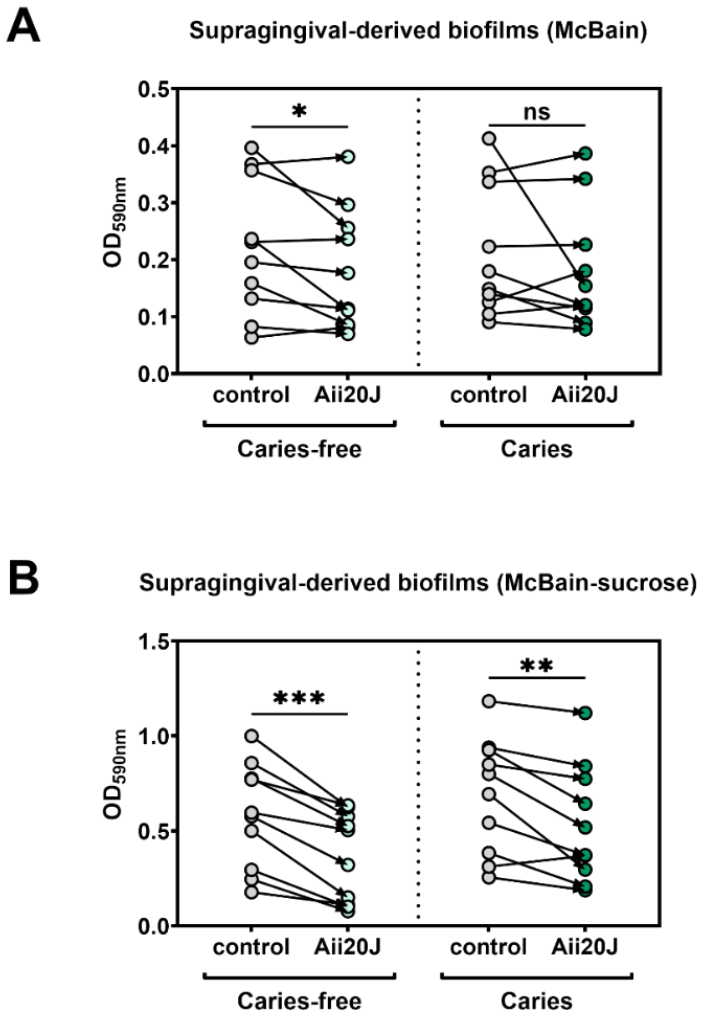


Figure 25. Graphical representation of paired *t*-tests comparing untreated and Aii20J treated biofilms. Samples were grown in McBain medium **(A)**, and in McBain supplemented with 0.2% sucrose **(B).** Before-after graphs represent values of biofilm quantification, assessed by CV assay (OD_{590nm}, y-axis), of untreated biofilms (grey dots) and Aii20J-treated biofilms (light and dark green dots). Arrows connect samples from the same patient. Samples are grouped according to the health status of the patient in caries-free (left part of the x-axis) and caries (right part of the x-axis). Statistical significance of paired *t*-tests between Aii20J-treated and untreated biofilms is displayed as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (paired *t*-tests, α = 0.05).

To investigate the microbial composition of the biofilms that responded to the Aii20J treatment with the greater biofilm mass reductions, gDNA was extracted from biofilms grown in McBain supplemented with sucrose, and the 16S rRNA of these samples was sequenced. Figure 26A shows the ten most relatively abundant genera identified in the biofilms generated in the presence of sucrose, either under exposure to the QQ enzyme Aii20J or as untreated controls.

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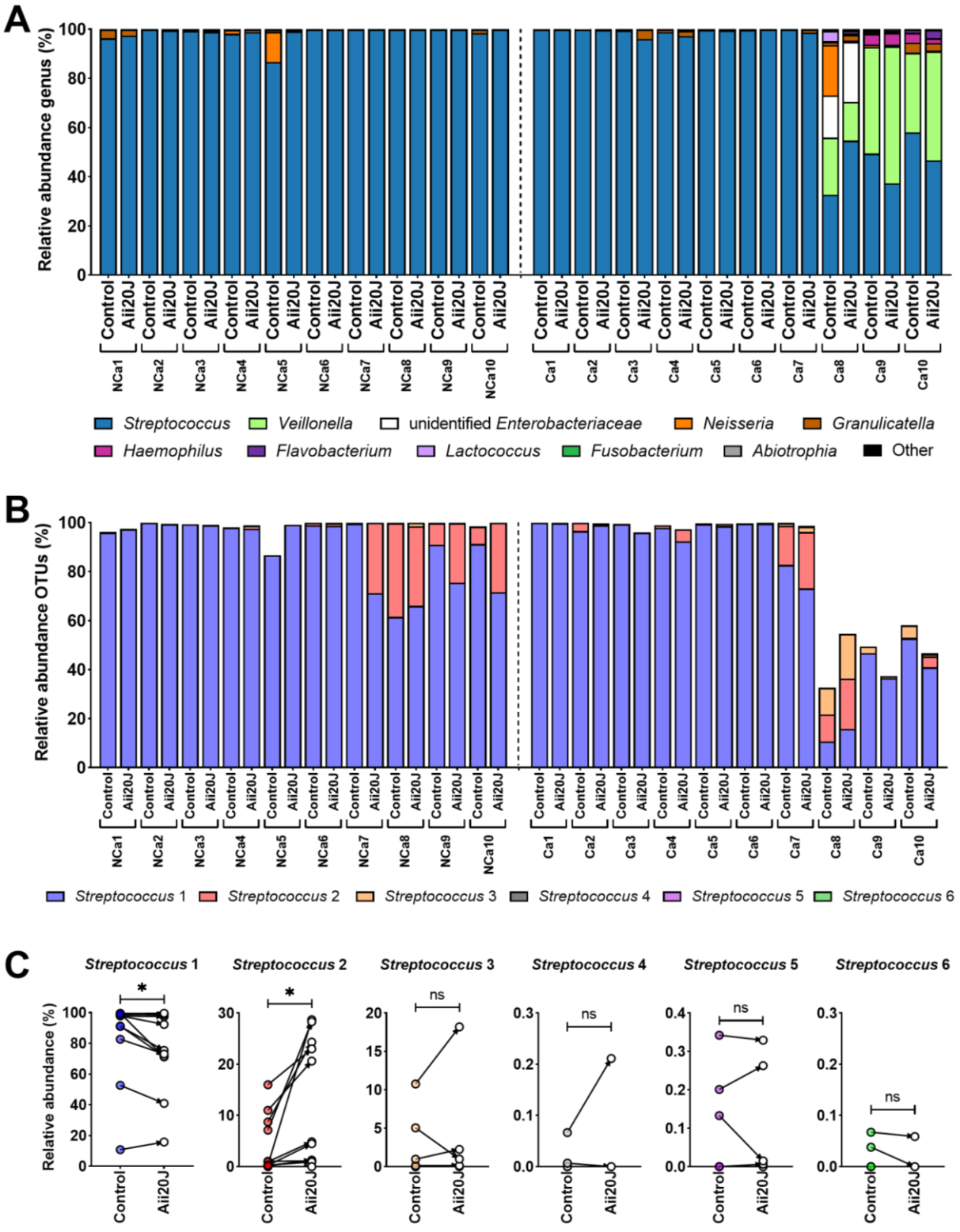


Figure 26. Relative abundance of the most abundant bacterial genera identified in *in vitro* biofilms obtained from supragingival biofilm samples. Supragingival samples were collected with curette from caries-free children (NCa) and children with caries (Ca). Biofilms were generated in the AAA model under exposure to the QQ enzyme Aii20J or as untreated controls. **A.** Relative abundance at the genus level is represented as the percentage of the 10 most relatively abundant bacterial genera identified in each sample (y-axis). Genera detected in lower relative

abundance appear under the category "Other". **B.** Relative abundance at the OTU level for members of the genus *Streptococcus*. **C.** Before-after graphs representing paired *t*-tests comparing untreated (coloured dots) and Aii20J treated biofilms (white dots) in terms of abundance of OTUs assigned to the genus *Streptococcus*. Arrows connect samples from the same patient. Statistical significance of paired *t*-tests between Aii20J-treated and untreated biofilms is displayed as *p ≤ 0.05 (paired *t*-tests, α = 0.05).

Overall, samples displayed a much lower diversity than the initial supragingival biofilms. The most abundant genus among the *in vitro* biofilms was *Streptococcus*, with relative abundances between 86.6 – 99.9%, followed by *Granulicatella* and *Neisseria*, with relative abundances of 0 – 4% and 0 – 12%, respectively (Figure 26A). The exceptions to this general tendency were biofilms Ca8, Ca9 and Ca10, generated from the samples of three patients with caries, which comprised *Streptococcus* relative abundances between 33 – 58%. In these samples, *Veillonella* was identified in a 16 – 56% relative abundance, a member of the family *Enterobacteriaceae* was identified only in Ca8 samples in a 17 – 24% relative abundance, and *Neisseria* was found in < 0.1 – 21% relative abundance (Figure 26A). Overall, biofilms from caries-free samples comprised between 2 – 6 identified genera (patients NCa1 – Nca3 and NCa7 – Nca10), with some samples reaching up to 24 genera (patient NCa5), whereas in most caries samples, there were around 10 – 16 genera (patients Ca1, Ca3 – Ca5, Ca8 – Ca10). When comparing the microbiome structures of untreated biofilms and biofilms grown under exposure to Aii20J, no significant differences were found either in caries-free or caries-derived biofilms (PCA, data not shown). However, examination of the taxonomical assignments at the OTU level revealed an interesting tendency among the OTUs belonging to the genus *Streptococcus*. We observed that one of the OTUs was significantly decreased in several Aii20J-treated biofilms that had responded to the enzyme by reducing their biofilm mass (*t*-test, α = 0.05) (Figure 26B,C). This OTU was labelled "*Streptococcus* 2". In parallel, a different OTU ("*Streptococcus* 1") appeared significantly increased in the same samples (Figure 26B,C). This substitution between such closely related gram-positives upon exposure to Aii20J seems to suggest the occurrence of a response to AHLs or AHL-like molecules produced by taxa present at very low relative abundances within these biofilms.

Taken together, our results show that Aii20J significantly reduces biofilms that are vastly dominated by gram-positives, specifically by *Streptococcus*. A study on the effect of Aii20J on biofilms of *S. mutans* revealed that this enzyme did not affect pure cultures of this bacterium (Muras et al., 2018). Thus, the effect observed in the present study is probably due to the surrounding environment of the polymicrobial biofilms generated. These results further

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support the use of Aii20J as an antibiofilm strategy and an alternative to antimicrobials for oral diseases while pointing towards the potential interactions of gram-positive taxa with AHLs or AHL-related compounds produced in polymicrobial contexts.

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CHAPTER 3 | Responses of *Enterococcus faecalis* biofilms to AHLs

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This chapter has been done in collaboration with the Department of Dental Medicine at Karolinska Institutet (SE), during a PhD research visit, under the supervision of Prof. G.N. Belibasakis. The last experimental section (section 3.3) has been done in collaboration with the CRETUS at the University of Santiago de Compostela (ES) in the laboratory of Prof. J. Lema.

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Background

Enterococcus faecalis is a facultative anaerobe, gram-positive bacterium that is commensal in the oral and gut microbiota of humans. Yet, outside of its niches, *E. faecalis* behaves as a major human opportunistic pathogen (García-Solache and Rice, 2019). Epidemiological reports point to the bacterium as a leading cause of nosocomial infections, including mostly urinary tract, surgical sites, endocardium and bloodstream infections (Peel et al., 2012; Brinkwirth et al., 2021). Besides, in oral medicine, *E. faecalis* is the most frequently retrieved taxon from recurring dental root infections, thereby highlighting the remarkable ability of the species to survive antimicrobial procedures (Siqueira et al., 2016; Bouillaguet et al., 2018; Manoil et al., 2020; Wong et al., 2021).

One crucial factor that allows *E. faecalis* to withstand harsh conditions and colonise multiple sites is its capacity to rapidly adhere to surfaces and form biofilms (Mohamed and Huang, 2007; Ran et al., 2015). Within biofilms, *E. faecalis* cells become protected from environmental stresses via a lower cellular metabolism and the production of an extracellular polymeric matrix (Stuart et al., 2006). As a result, bacteria embedded within biofilms are classically estimated to be approximately 1000-fold less susceptible to antimicrobials, which is clinically troublesome when considering that biofilms account for an estimated 65 to 80% of infections that require hospitalisation (Costerton et al., 1999; Lewis, 2001). Therefore, a better understanding of the factors underlying biofilm formation, and of ways to tackle these infections, remains imperative.

Initiation of bacterial biofilm formation is, in many cases, regulated by quorum sensing (QS), i.e., a cell-density sensing mechanism regulated by the accumulation of signalling molecules in the vicinity of bacterial cells (Parsek and Greenberg, 2005). The binding of these molecules to their cognate receptors induces a series of signal transductions that coordinate gene expression in the microbial community. It is classically described that low molecular weight oligopeptides are involved in gram-positive signalling, as they easily diffuse through their peptidoglycan cell wall, whereas gram-negative bacteria rely on acyl-homoserine lactones (AHLs) (Fuqua and Greenberg, 2002). AHLs comprise a homoserine lactone (HSL) ring connected to an acyl chain of variable length, which renders the molecule hydrophobic and hence able to cross gram-negatives' outer membrane (Fuqua and Greenberg, 2002). Nonetheless, recent evidence suggests that these intergeneric signalling boundaries may not be so strict. Specifically, several gram-positives, including *Enterococcus faecalis, Listeria*

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monocytogenes and *Staphylococcus sciuri*, were shown to increase their biofilm formation upon exposure to AHLs (Bensalem et al., 2014; Naik et al., 2017, 2018). Also, exposure of *Staphylococcus aureus* to an oxo-substituted AHL (OC_{12}-HSL) was shown to decrease its expression of exotoxins, fibronectin adhesins, and the *agr* virulence-associated regulon, while up-regulating an immunoglobulin-binding protein (Qazi et al., 2006). Besides, previous results pointed to the role of AHLs or AHL-like molecules in gram-positive dominated biofilms (see Chapters 1 and 2). Whereas these reports suggest an effect of AHLs on gram-positives as well, the extent of the effect of these molecules on biofilm formation and the differential regulation of virulence-associated traits remains poorly understood.

Therefore, we aimed to examine the effects of different AHLs on the growth, transcriptional regulations and proteomic profiles of *E. faecalis* biofilms. Specifically, biofilms from different *E. faecalis* strains were quantified after exposure to AHLs by CV assay. Furthermore, *E. faecalis* cells embedded in the biofilm were observed by confocal-laser scanning microscopy in combination with SYTO 9 / propidium iodide (PI) staining. Additionally, the differential expression of ten key player genes involved in peptide-mediated QS, biofilm formation, and membrane stress responses was assessed by reverse-transcription and quantitative PCR (RT-qPCR). Finally, the proteomes of *E. faecalis* biofilms exposed to AHLs were investigated using label-free quantitative proteomics.

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Results and discussion

1. Optimisation of *E. faecalis* biofilm generation

Optimisation of the biofilm generation methodology was done using *E. faecalis* ATCC 29212. This collection strain was used as a reference throughout several experiments due to the complete annotation of its genome in NCBI's genome database (National Library of Medicine (US), 2012). Biofilms were generated from *E. faecalis* ATCC 29212 suspensions in a modification of the Amsterdam Active Attachment model (AAA model) (Exterkate et al., 2010; Muras et al., 2020a) assembled with glass coverslips. In addition, biofilms were generated in a 24-well plate model, quantifying the biofilm formed onto the bottom and walls of the wells. Figure 27 shows absorbance values (OD_{595nm}) of biofilms generated in the AAA model (Figure 27A) and 24-well polystyrene plates (Figure 27B) and quantified by CV assay.

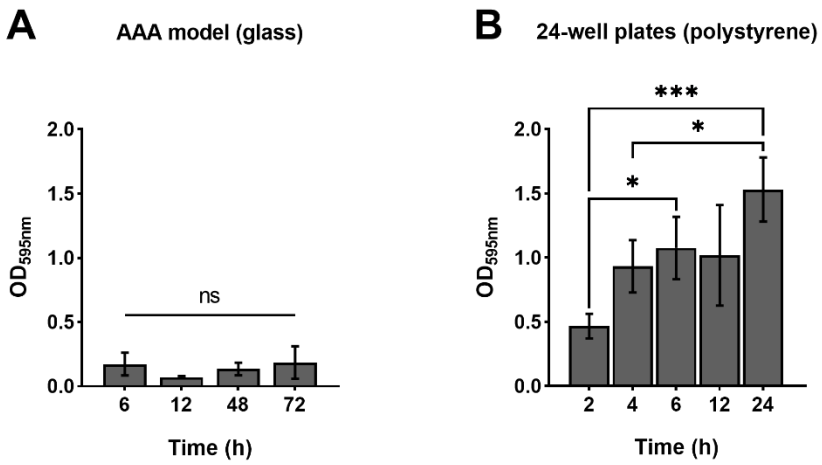


Figure 27. Biofilm generation in *E. faecalis* ATCC 29212 in different cultivation systems and at different incubation times. *E. faecalis* ATCC 29212 biofilm generation in the AAA model assembled with glass coverslips **(A)** and onto the bottom of 24-well polystyrene plates **(B).** Histograms represent the biofilm mass assessed by CV assays. The y-axis shows the amount of CV measured by optical density readings at 595 nm (OD_{595nm}). Bars show biofilms assessed at different incubation times (x-axis). Bars display mean values of 3 replicates, and whiskers represent SD. Statistical significance of pairwise comparisons between biofilm quantification values at each incubation time is displayed as *$p \leq 0.05$, ***$p \leq 0.001$ (ANOVA and Tukey's test, $\alpha = 0.05$). ns = not significant.

E. faecalis biofilms grown on the AAA model showed poor attachment to the glass coverslips. The amount of biomass was low throughout different incubation times, reaching less than 0.2 absorbance at OD_{595nm}, as assessed by CV assay (Figure 27A). The AAA model relies on the active attachment of bacteria to a surface that is vertically submerged in the culture medium. *E. faecalis* is a non-motile, facultative anaerobe that in *in vitro* static cultures tends to

settle on the bottom of the wells or tubes, which could explain the reduced attachment observed in the AAA model regardless of the incubation time (Figure 27A). Nevertheless, when *E. faecalis* suspensions were grown in 24-well plates, we observed the attachment of biofilms onto the bottom of the wells (Figure 27B). The attached biomass could be detected even after 2 hours of incubation, and it increased with the incubation time, reaching absorbance values of 1.0 at 4 – 12 hours of incubation and 1.5 at 24 hours of incubation (Figure 27B). These results prompted the use of the 24-well plate system for subsequent experiments, and 6 hours of incubation were chosen to represent early biofilm stages.

2. Effect of AHL exposure on *E. faecalis* biofilm generation and gene expression

2.1. Effect of AHL exposure on biofilm generation

The effect of AHL exposure on biofilm was assessed using five *E. faecalis* strains. Suspensions from each strain were exposed to either a combination of either short-chain AHLs (comprising C_4-HSL, OC_4-HSL, C_6-HSL, OC_6-HSL, and C_8-HSL, to a concentration of 1 μM for each AHL), or long-chain AHLs (comprising C_{10}-HSL, C_{12}-HSL, C_{14}-HSL, and C_{18}-HSL, to a concentration of 1 μM for each AHL). This clustering of AHLs, although mostly didactic, relied on an ecological rationale, as evidence shows that the length of AHLs conditions their species-specific recognition in gram-negative taxa (Waters and Bassler, 2005). A typical example is the bacterium *Chromobacterium violaceum*, in which short-chain (C_4 – C_8) AHLs induce violacein production in certain strains and long-chain AHLs (C_{10} – C_{16}) antagonise this effect, whereas the opposite relationship between long- and short-chain AHLs is observed in other strains (McClean et al., 1997; Morohoshi et al., 2008). Nevertheless, it is to be noted that this approach precludes the discrimination of the effect of each AHL.

Figure 28 shows the CV quantification of *E. faecalis* biofilms following exposure to either short- or long-chain AHLs. The five strains investigated displayed varying abilities to produce biofilms. Upon AHL exposure, all strains exhibited a tendency to increase biomass production. Specifically, the reference strain ATCC 29212 significantly increased its biofilm formation by 36% upon exposure to long chains AHLs. Significant increases in biomass were also observed in strains UmID4 and UmID5. More precisely, UmID4 displayed 33% and 42% biomass increase following exposure to short- and long-chain AHLs respectively, whereas UmID5 exhibited a 40% increase after exposure to long-chain AHLs, whereas short-chain AHLs had no effect on biofilm mass. These results aligns with those of Bensalem et al., who

described even higher increases in *E. faecalis* biofilm upon exposure to C_6-HSL (72%) and C_8-HSL (54%) (Bensalem et al., 2014). In addition, Naik et al. also described similar biofilm increases to the ones observed in the present study on gram-positive bacteria exposed to C_6-HSL, with reports of a 47% biofilm increase in *Listeria monocytogenes* (Naik et al., 2017), and of 24% in *Staphylococcus sciuri* (Naik et al., 2018).

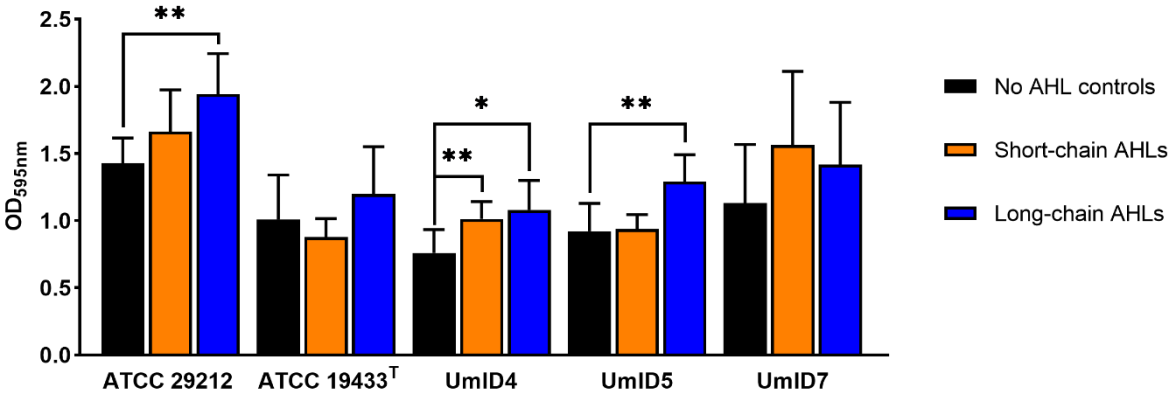


Figure 28. Effect of AHL mixes on *E. faecalis* biofilm formation. Histograms represent the amount of biofilm formed by each *E. faecalis* strain, assessed by CV assays. The y-axis shows the amount of CV measured by optical density readings at OD_{595nm}. Black bars show biofilms exposed to BHI with the corresponding fraction of acetonitrile (no AHL controls), orange bars show biofilms exposed to the short-chain AHLs mix, and blue bars show biofilms exposed to the long-chain AHLs mix. Bars display mean values of three independent experiments performed in triplicate, and whiskers represent SD (n = 9). Statistical significance of pairwise comparisons between each AHL mix (short- or long-chains) and the no AHL control is displayed as *p ≤ 0.05, **p ≤ 0.01 (Mann-Whitney, α = 0.05).

In parallel to biomass assessments, the growth curves of the five *E. faecalis* strains were monitored for the same duration of the biofilm generation experiments in shaken cultures in 96-well plates (Figure 29A – E). As neither short- nor long-chain AHLs influenced the planktonic growth of none of the strains investigated, we hypothesise that the observed increases in biofilm mass can be attributed to a specific increase in the number of biofilm cells, and discard an increase in total cellular growth.

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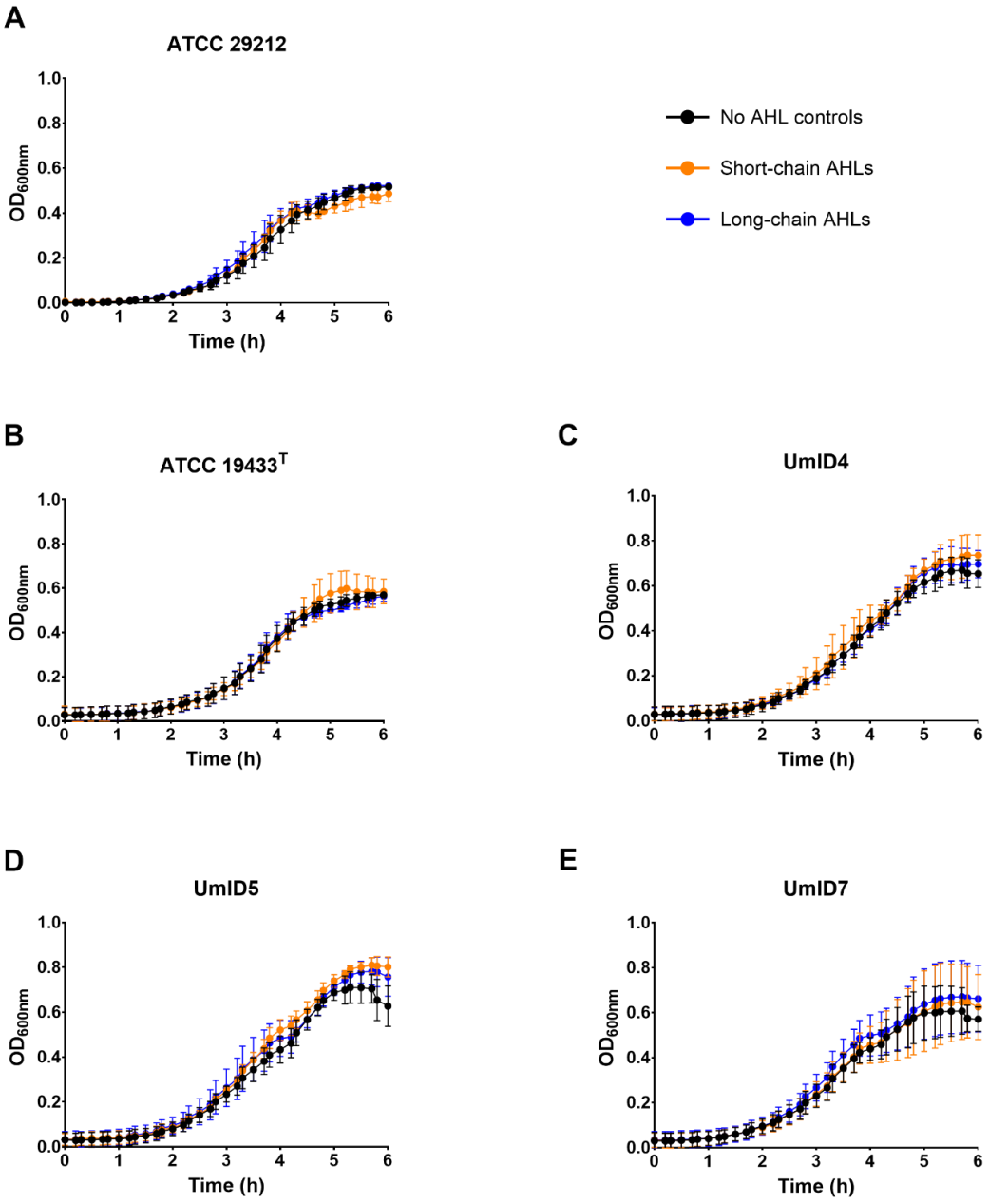


Figure 29. Planktonic growth curves of *E. faecalis* cultures exposed to AHL mixes. Graphs represent the growth of each *E. faecalis* strain; ATCC 29212 **(A)**, ATCC 19433^T **(B)**, UmID4 **(C)**, UmID5 **(D)**, and UmID7 **(E).** Cultures were grown at 37 ˚C with orbital shaking in 96-well plates. Growth was monitored by optical density readings at OD_{600nm} (y-axis). Black dots show suspensions exposed to BHI only (no AHL controls), whereas orange and blue dots show cultures exposed to short- and long-chain AHLs, respectively. OD readings were acquired every 10 min. Each time-point measurement represents the mean of 2 independent experiments performed in triplicate, and whiskers show SD (n = 6). Growth curves were analysed for statistically significant differences with one-way ANOVAs and Dunnett's multiple comparisons tests (α = 0.05).

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To confirm the effect of the AHL mixes on biofilms of *E. faecalis*, the strain ATCC 29212 was further analysed by confocal microscopy. Figure 30 shows confocal microscopy observations of *E. faecalis* ATCC 29212 biofilms exposed to either short- or long-chain AHLs and stained with a combination of SYTO 9 and PI. Most cells appeared intact (SYTO 9-positive) and aggregated in microcolonies, whereas membrane-damaged cells (PI-positive) were only sporadically observed independently of the presence or absence of AHLs (Figure 30A). This observation aligns with the short maturation stage of the biofilms assessed. Measurements of biofilm-covered areas revealed that biofilms not exposed to AHLs extended over 48 ± 15% of the field of view (FoV), whereas biofilms exposed to long-chain AHLs displayed a significantly increased coverage to 72 ± 5% of the FoV (Figure 30B). These results further confirmed that AHLs promote biofilm formation. AHL-mediated QS is a known factor driving biofilm formation in gram-negative taxa (Mayer et al., 2020; Muras and Otero, 2020). In addition, *E. faecalis* is commonly found in polymicrobial infections in close contact with gram-negative bacteria able to produce AHLs (Ch'ng et al., 2019). Hence, in such settings, *E. faecalis* is potentially exposed to AHLs and could establish a beneficial association between *E. faecalis* and the AHL-producing gram-negative taxa. In this association, the use of AHLs by *E. faecalis* could represent a competitive strategy to outgrow other bacteria or even a mutualistic strategy, with the promotion of *E. faecalis* biofilm formation offering a shield against environmental stresses for neighbouring bacteria. Studying dual-species biofilms of *E. faecalis* and AHL-producer gram-negative taxa would be interesting to gain knowledge regarding these potential interactions. The response of *E. faecalis* strain UmID4 to both short- and long-chain AHLs suggests the presence of a promiscuous receptor or that of a receptor for other molecules that can interact with AHLs. The existence of such receptors in gram-positive taxa has also been suggested following the results obtained in Chapters 1 and 2 of the present thesis. However, so far, no receptors of this kind have been described in gram-positive taxa.

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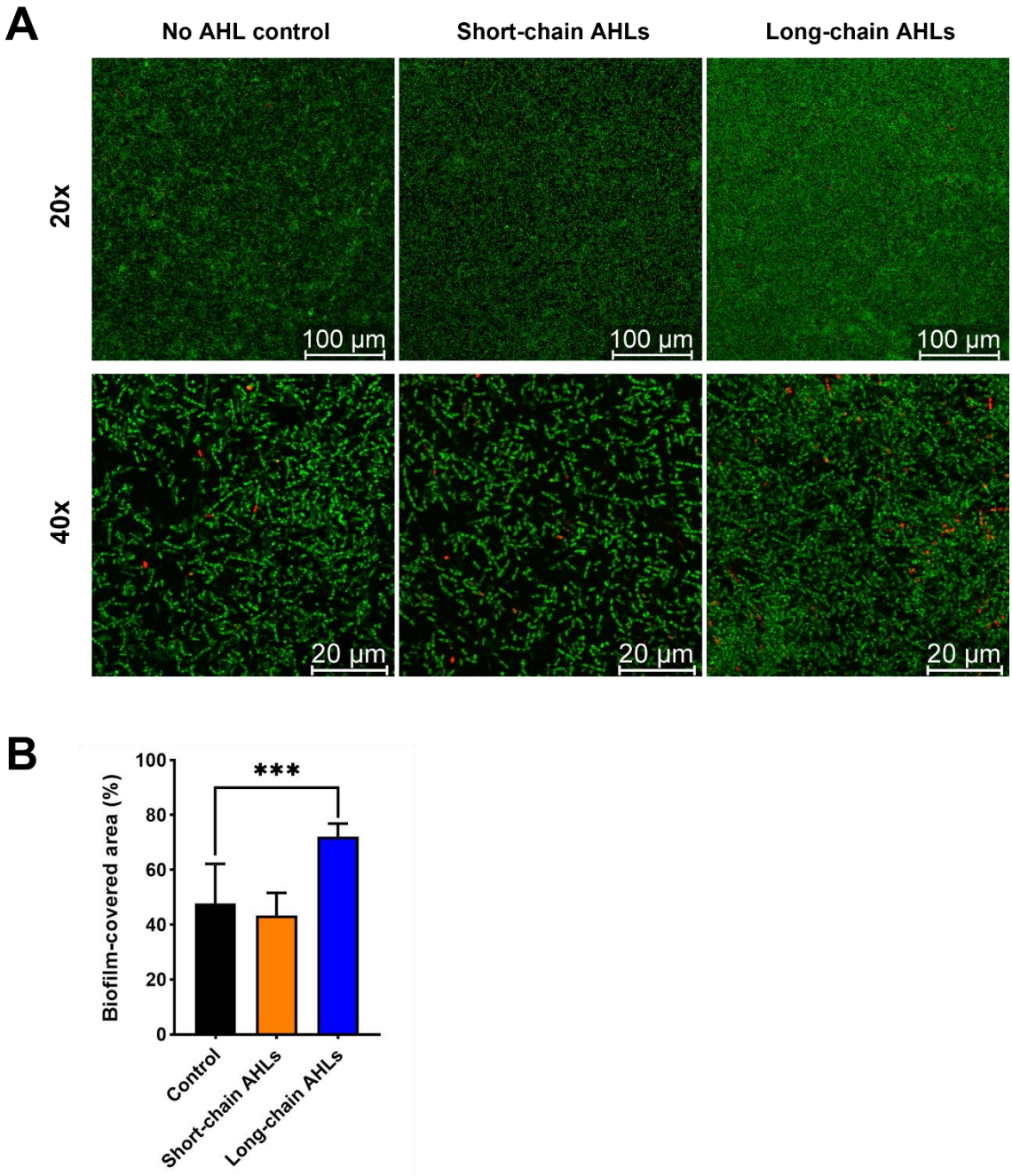


Figure 30. Confocal-laser scanning microscopy visualisation of *E. faecalis* biofilms exposed to either short- or long-chain AHLs. A. Representative microphotographs of *E. faecalis* ATCC 29212 biofilms grown with no AHL exposure (left), and in the presence of either short- (middle) or long-chain AHLs (right). The upper photomicrographs display fields of view observed at 20x magnification, and lower photomicrographs, at 40x magnification. Scale bars represent 100 µm on 20x photomicrographs, and 20 µm on 40x photomicrographs. *E. faecalis* cells stained with SYTO 9 appear green, and cells stained with PI appear red. **B.** Histograms represent the area covered by *E. faecalis* biofilms. The y-axis expresses the percentage of biofilm-covered area relative to the total field of view (n = 8 fields at 20x). Statistical significance of pairwise comparisons between each AHL mix (short- or long-chains) and the no AHL control is displayed as ***$p \leq 0.001$ (t-tests, $\alpha = 0.05$).

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2.2. Differential gene expression in E. faecalis biofilms exposed to AHLs

To gain deeper insights into the effects of AHLs on *E. faecalis* biofilms, the differential expression of ten key transcripts involved in quorum sensing, biofilm formation and membrane stress responses was analysed. The ten transcripts were grouped into three categories representative of their functions: peptide-mediated QS-related genes (Figure 31), biofilm-associated genes (Figure 32), and genes associated to membrane-stress responses (Figure 33). Down-regulations lower than 0.5-fold and up-regulations higher than 2-fold were considered biologically relevant differences in transcription levels. Overall, one observed that gene regulation in response to AHLs was heterogeneous among the strains investigated (Figure 31, Figure 32, and Figure 33), which can be explained by the high genome plasticity characterising *E. faecalis* (Palmer et al., 2012). This is translated into multiple possible combinations of genetic elements among strains of *E. faecalis*, which can result in a strain-specific differential regulation of gene expression. This is further exemplified by the identification of three out of the ten genes of interest (*cylA*, *cylM* and *asa1*) in only one of the five strains investigated; the strain ATCC 29212 (Figure 31B, Figure 32C). Hence, the investigation of five different *E. faecalis* strains allowed us to examine responses at gene level that could have been concealed by the use of a single strain.

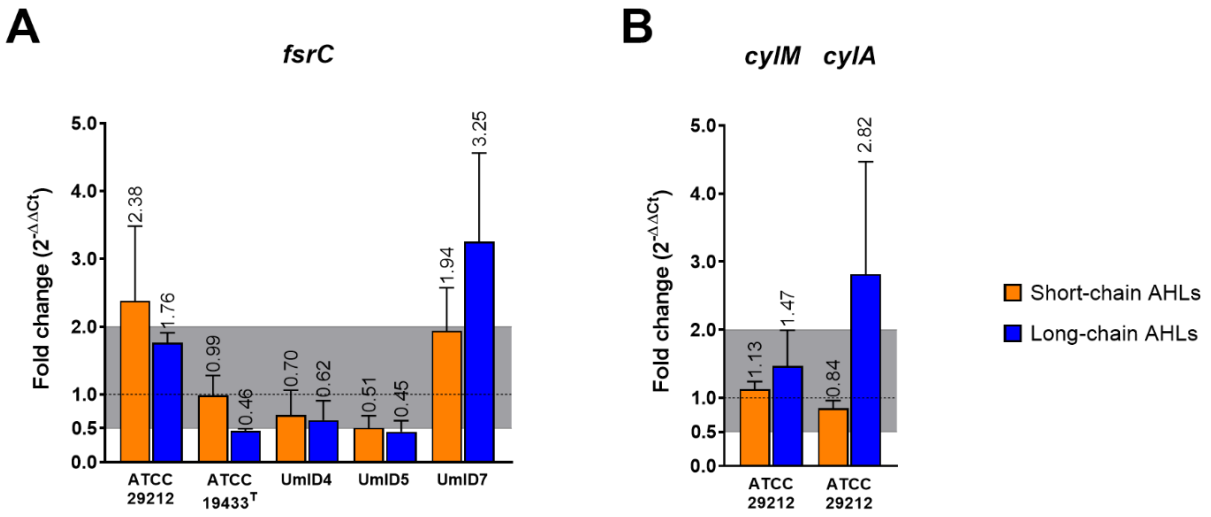


Figure 31. Differential expression of QS-related genes in biofilms of *E. faecalis* exposed to AHLs. Histograms display the transcription levels of *fsrC* **(A),** and *cylM* and *cylA* **(B)** as fold-changes calculated by the $2^{-\Delta\Delta Ct}$ algorithm (y-axis). The genes *cylM* and *cylA* **(B)** were identified only in strain ATCC 29212. Differential expression from biofilms exposed to short-chain ALHs is displayed by orange bars and that of biofilms exposed to long-chain ALHs by blue bars. Bars show mean fold-change values, and whiskers represent SEM (*n* = 4 biological replicates). The

value of each mean is additionally indicated above whiskers. The dotted line at y = 1 defines the expression level of each gene in the calibrator condition, normalised to the expression of the 16S rRNA gene. The grey area marks the range of changes in transcript levels not considered biologically relevant. Statistical comparisons between conditions were assessed with Mann-Whitney tests (α = 0.05).

Among the QS-related genes investigated, *fsrC* was up-regulated by 2.4-fold in strain ATCC 29212 after exposure to short-chain AHLs. Similarly, *fsrC* displayed a 3.3-fold up-regulation in strain UmID7 after exposure to long-chain AHLs (Figure 31A). The *fsrC* transcript encodes for the histidine kinase of the *fsr* operon, the main QS system in *E. faecalis*. FsrC recognises and binds to the gelatinase-biosynthesis activating pheromone (GBAP), which acts as an autoinducer peptide. The activation of the response regulators of the *fsr* operon controls the expression of important virulence factors in *E. faecalis* (Qin et al., 2001; Gaspar et al., 2009). The *fsr* operon is a homologue of the accessory gene regulator (*agr*) operon of *S. aureus* (Qin et al., 2001). Inversely to the results presented here, exposure of *S. aureus* to the AHL OC_{12}-HSL down-regulates the expression of the *agr* operon (Qazi et al., 2006). Nevertheless, it is worth mentioning that spontaneous degradation in aqueous media of oxo-substituted long-chain AHLs, such as OC_{12}-HSL, can result in the formation of tetramic acid derivatives (Kaufmann et al., 2005). These tetramic acids have been described to present antibacterial activity against gram-positive taxa (Kaufmann et al., 2005). In the present study, none of the long-chain AHLs included in the mixes had oxo-substitutions in their acyl chains to avoid potential biases in the interpretation of the results obtained upon AHL exposure. Taken together, this evidence points to a potential effect of AHLs on *agr* and *agr*-like systems, although it remains to be investigated whether the differential responses are due to the specificity of each system.

The second QS system investigated here is the *cyl* operon, which regulates the production of the bacteriocin cytolysin sensing the extracellular concentration of one of the cytolysin subunits (Coburn and Gilmore, 2003). Among the genes studied here, *cylM*, encodes a lanthionine synthetase responsible for the post-translational modification of the subunits of the cytolysin, and *cylA* encodes an extracellular serine protease that activates the cytolysin subunits (Ali et al., 2017). These two genes, which can be either encoded in the *E. faecalis* chromosome or in a plasmid, were only identified in strain ATCC 29212 (Figure 31B). Similarly to the observed results with the *fsrC* transcript, our data show a 2.8-fold up-regulation of *cylA* in the strain ATCC 29212. Together these data indicate that exposure to long-chain AHLs especially, appears to activate quorum sensing operons and up-regulate virulence-

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associated factors, at least in some *E. faecalis* strains. Whereas the mechanisms underlying these observations cannot be inferred from our data, it may be relevant to mention that GBAP, the natural autoinducer of the *fsr* operon, also exhibits a lactone ring structurally similar to that of AHLs (Cook and Federle, 2014). A potential cross-reactivity between GBAP and other lactone-containing molecules has been previously reported and may, therefore, also underlie current observations (Nakayama et al., 2013).

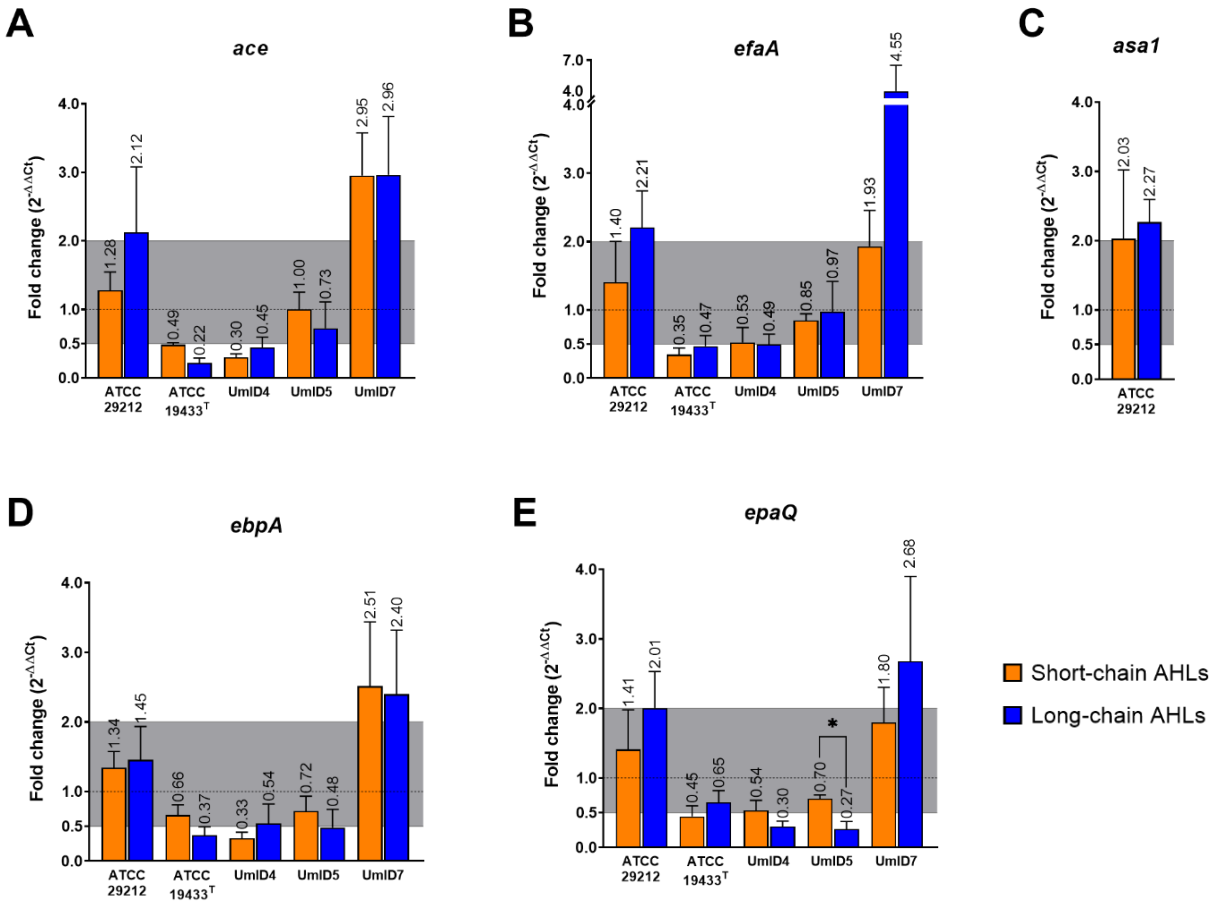


Figure 32. Differential expression of biofilm-related genes in biofilms of *E. faecalis* exposed to AHLs. Histograms display the transcription levels of *ace* **(A)**, *efaA* **(B)**, *asa1* **(C)**, *ebpA* **(D)**, and *epaQ* **(E)** as fold-changes calculated by the $2^{-\Delta\Delta Ct}$ algorithm (y-axis). Expression levels of *efaA* are shown on a split y-axis that covers fold-changes from 0 to 4 on its lower segment and from 4 to 7 on its upper segment. The gene *asa1* **(C)** was identified only in strain ATCC 29212. Differential expression from biofilms exposed to short-chain ALHs is displayed by orange bars and that of biofilms exposed to long-chain ALHs by blue bars. Bars show mean fold-change values (*n* = 4 biological replicates) and whiskers represent SEM. The value of each mean is additionally indicated above whiskers. The dotted line at y = 1 defines the expression level of each gene in the calibrator condition, normalised to the expression of the 16S rRNA gene. The grey area marks the range of changes in transcript levels not considered biologically relevant. Statistical comparisons between conditions are displayed as *p ≤ 0.05 (Mann-Whitney tests, α = 0.05).

Current gene expression assessments also revealed several biologically relevant up- and down-regulations among genes encoding adhesins, pili and enzymes involved in biofilm formation. Among them, the collagen adhesin-encoding *ace* appeared 2.1-fold up-regulated in strain ATCC 29212 after exposure to long-chain AHLs (Figure 32A), corroborating phenotypic measurements obtained in CV assay (see Figure 28). More important up-regulations were observed in strain UmID7, with 3-fold up-regulations after exposure to either short- or long-chain AHLs (Figure 32A), but with no phenotypic effect upon exposure to the same AHLs (see Figure 28). Contrastingly, the *ace* transcript displayed down-regulations in strains ATCC 19433^{T} and UmID4 following AHL exposure. The endocarditis antigen A (*efaA*), encoding another adhesin of *E. faecalis*, showed relevant up-regulations upon exposure to long-chain AHLs, reaching 2.2-fold in strain ATCC 29212, and 4.6-fold in strain UmID7 (Figure 32B). The adhesins *ace* and *efaA* are strongly related to pathogenicity and initial adhesion. The transcript *ace* encodes a collagen-binding protein displayed on *E. faecalis* membranes under the indirect control of *fsr*-mediated QS, contributing to biofilm dispersal (Rich et al., 1999; Pinkston et al., 2011). In addition, expression of *efaA* has also been proposed to be controlled by the *fsr* operon at early growth stages (Bourgogne et al., 2006). The observed up-regulation of *fsrC* and these two adhesins seems to imply an increased virulence in *E. faecalis* strains, pointing to AHLs as potential virulence-inducing cues.

Among the three other biofilm-associated genes, the aggregation substance, encoded by *asa1*, was only identified in strain ATCC 29212, in which it was up-regulated upon exposure to both short- (2-fold) and long-chain AHLs (2.3-fold) (Figure 32C). The pili-encoding transcript *ebpA* showed relevant up-regulations in strain UmID7 in response to short- (2.5-fold) and long-chain AHL mixes (2.4-fold), whereas down-regulations were observed in strains ATCC 194333^{T}, UmID4 and UmID5 (Figure 32D). Finally, the hypothetical glycosyl transferase *epaQ* exhibited 2- and 2.7-fold up-regulation in strains ATCC 29212 and UmID7 respectively, in response to long-chain AHLs. Inversely, exposure to long-chain AHLs in UmID4 and UmID5 rather down-regulated *epaQ* expression to 0.3-fold in both strains (Figure 32E). These three genes are closely related to virulence in *E. faecalis*. The aggregation substance *asa1* is a surface protein implicated in adhesion to eukaryotic cells and plasmid transfer, and in addition, is regulated by cell-density dependent mechanisms (Galli et al., 1990; Ali et al., 2017; García-Solache and Rice, 2019). The pili subunit *ebpA* is expressed during infection (Nallapareddy et al., 2006). The *epaQ* transcript has been demonstrated to be essential

for the normal biofilm architecture in *E. faecalis* (Korir et al., 2019). The observed up-regulations indicate a potential role of AHLs as virulence-inducing molecules in *E. faecalis* strains ATCC 29212 and UmID7. Nevertheless, the differential regulations observed among strains did not correlate with the observed biofilm phenotypes, suggesting that a larger network of genes may be altered in *E. faecalis* upon AHL exposure. This finding also emphasises the importance of assessing several strains within the same species, especially when high genome plasticity has been described in the bacterium.

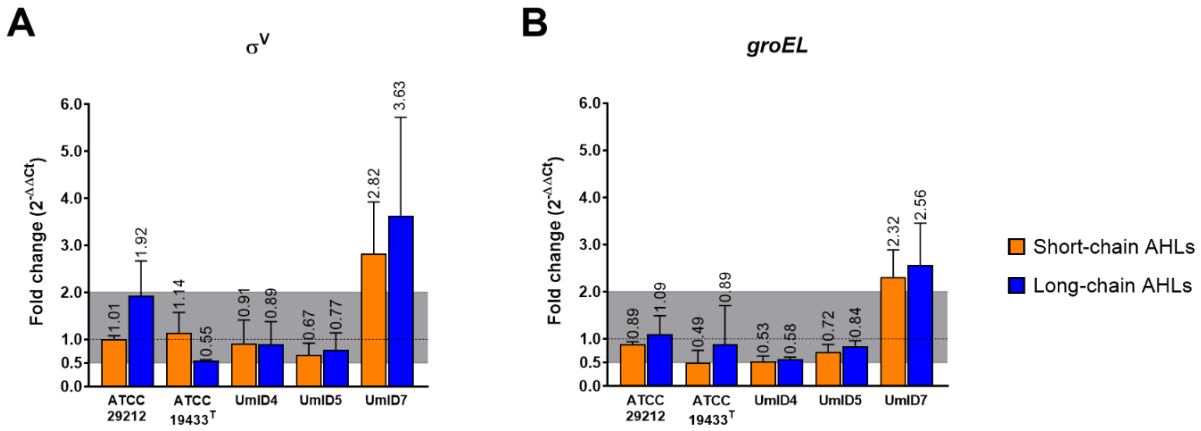


Figure 33. Differential expression of membrane-stress-related genes in biofilms of *E. faecalis* exposed to AHLs. Histograms display the transcription levels of σ^V **(A),** and *groEL* **(B)** as fold-changes calculated by the $2^{-\Delta\Delta Ct}$ algorithm (y-axis). Differential expression from biofilms exposed to short-chain ALHs is displayed by orange bars and that of biofilms exposed to long-chain ALHs by blue bars. Bars show mean fold-change values, and whiskers represent SEM (*n* = 4 biological replicates). The value of each mean is additionally indicated above whiskers. The dotted line at y = 1 defines the expression level of each gene in the calibrator condition, normalised to the expression of the 16S rRNA gene. The grey area marks the range of changes in transcript levels not considered biologically relevant. Statistical comparisons between conditions were assessed with Mann-Whitney tests (α = 0.05).

Among genes related to membrane-stress responses, the extra-cytoplasmic sigma-factor σ^V appeared relevantly up-regulated in strain UmID7 upon exposure to both short- (2.8-fold) and long-chain AHLs (3.6-fold) (Figure 33A). Similarly, the chaperon *groEL* was up-regulated in strain UmID7 after exposure to both short- (2.3-fold) and long-chain AHLs (2.6-fold) (Figure 33B). It has already been suggested that an AHL, OC_{12}-HSL, may perturb membrane function in the gram-positive *S. aureus* (Qazi et al., 2006). The same group described a high affinity of OC_{12}-HSL for *S. aureus* membranes, suggesting the interaction with specific membrane domains (Qazi et al., 2006). The sigma-factor σ^V and the chaperone *groEL* have been linked to membrane-stress responses in *E. faecalis* (Laport et al., 2001; Benachour et al., 2005; Manoil

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et al., 2022). In the present study, the up-regulation of their transcriptional levels in strain UmID7 upon exposure to AHLs seems to indicate that AHLs are perceived as a stressor and may suggest an interaction of these molecules with *E. faecalis* membranes.

3. Effect of individual AHLs on *E. faecalis* biofilm generation and proteomic profile

3.1. Effect of individual AHLs exposure on biofilm generation

The use of AHL mixes may preclude discerning the effect of each AHLs. Hence, the use of individual AHLs in this section aims to assess whether the responses observed in biofilm generation and gene expression are different under exposure to single AHLs. Similarly to the previous section, *E. faecalis* ATCC 29212 biofilms were generated in 24-well plates, in BHI, during 6 hours of incubation time. Figure 34 displays quantification of biofilms exposed to individual AHLs. Strain ATCC 29212 significantly increased its biofilm formation by 50% upon exposure to C_{18}-HSL (Mann-Whitney tests, $\alpha = 0.05$). Meanwhile, exposure to C_{10}-HSL induced an increase in biomass that was not statistically significant (Figure 34).

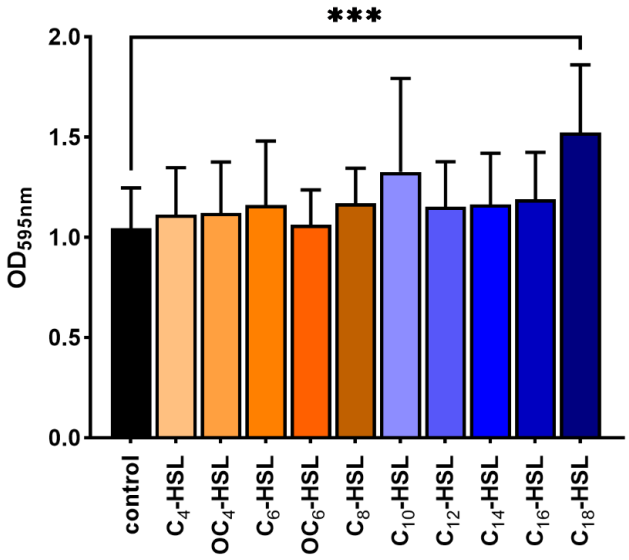


Figure 34. Effect of individual AHLs on *E. faecalis* ATCC 29212 biofilm formation at 6 hours of incubation. Histograms represent the amount of biofilm mass assessed by CV assays. The y-axis shows the amount of CV measured by optical density readings at 595 nm. Black bars show biofilms exposed to BHI only (control), and coloured bars show biofilms exposed to AHLs (x-axis). Bars display mean values of 4 independent experiments performed in triplicate, and whiskers represent SD (n = 12). Statistical significance of pairwise comparisons between each AHL and the no AHL control is displayed as ***$p \leq 0.001$ (Mann-Whitney tests, $\alpha = 0.05$).

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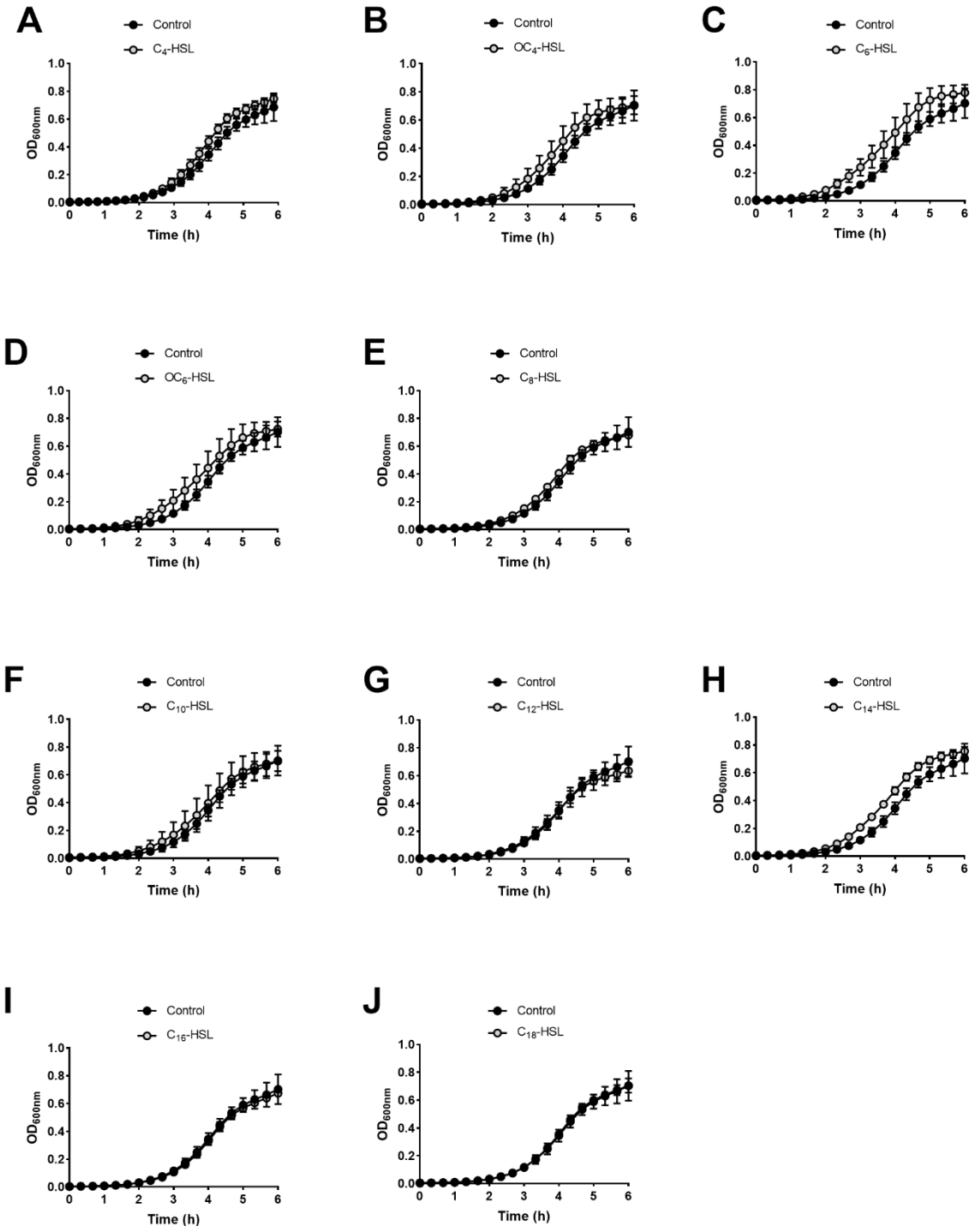


Figure 35. Planktonic growth curves of *E. faecalis* cultures exposed to individual AHLs. Graphs represent the growth of *E. faecalis* ATCC 29212 monitored in time by optical density readings at 600 nm (y-axis). Black dots show suspensions exposed to BHI only (control). White dots show suspensions exposed to AHLs; C_4-HSL **(A),** OC_4-HSL **(B),** C_6-HSL **(C),** OC_6-HSL **(D),** C_8-HSL **(E),** C_{10}-HSL **(F),** C_{12}-HSL **(G),** C_{14}-HSL **(H),** C_{16}-HSL **(I),** and C_{18}-HSL **(J).** OD readings were acquired every 15 min. Each time-point measurement represents the mean of 2 independent experiments performed in triplicate, and whiskers show SD (n = 6). Growth curves were analysed for statistically significant differences with one-way ANOVAs and Dunnett's multiple comparisons tests (α = 0.05).

In parallel to biofilm formation assays, the effect of exogenous AHLs on planktonic growth was assessed by optical density measurements under the same culture conditions and incubation time (Figure 35). None of the AHLs tested altered the planktonic growth of strain ATCC 29212. As seen under exposure to AHL mixes, C_{18}-HSL appears to specifically increase the number of biofilm cells, as it does not affect the planktonic growth of *E. faecalis* ATCC 29212 cultures.

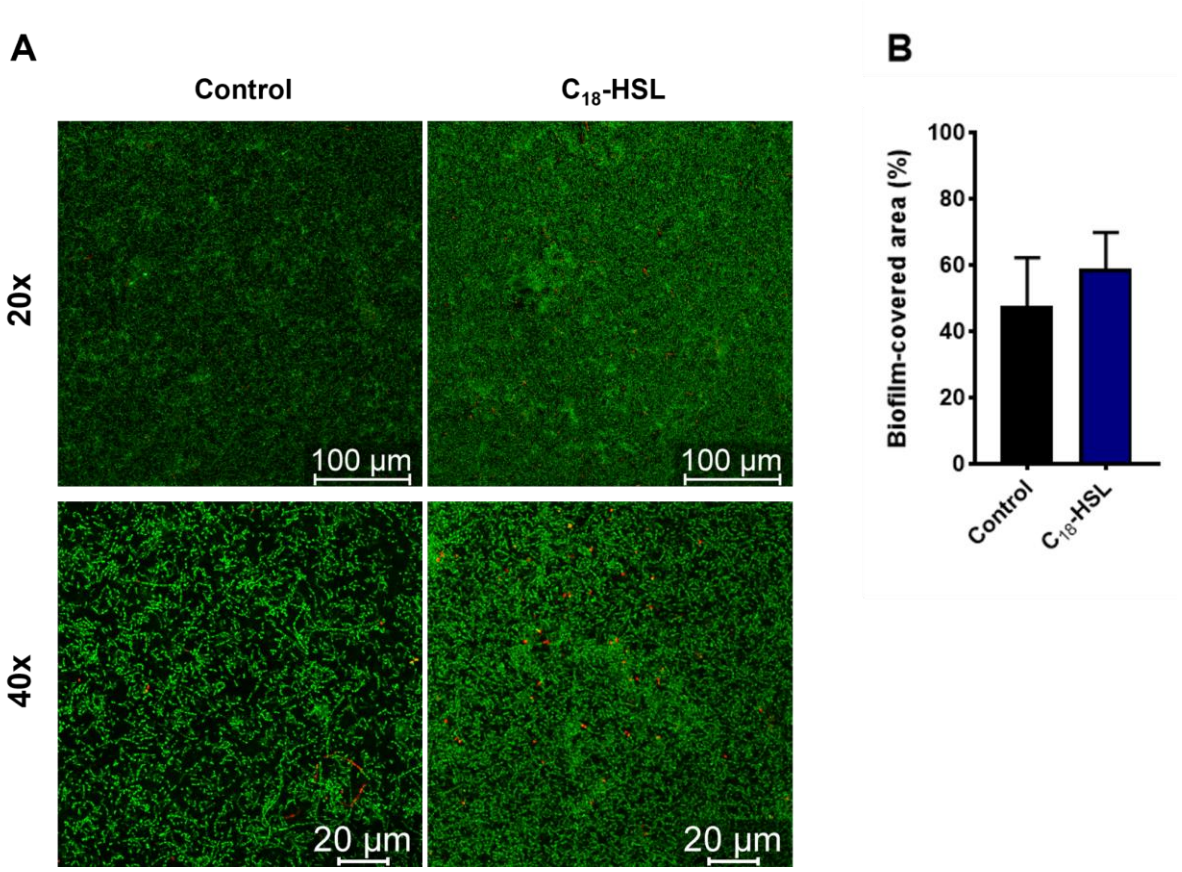


Figure 36. Confocal-laser scanning microscopy visualisation of *E. faecalis* biofilms. A. Representative microphotographs of *E. faecalis* ATCC 29212 biofilms grown with no AHL exposure (left), and in the presence of C_{18}-HSL (right). The upper photomicrographs display fields of view observed at 20x magnification, and lower photomicrographs, at 40x magnification. Scale bars represent 100 µm on 20x photomicrographs, and 20 µm on 40x photomicrographs. *E. faecalis* cells stained with SYTO 9 appear green, and cells stained with PI appear red. **B.** Histograms represent the area covered by *E. faecalis* biofilms. The y-axis expresses the percentage of biofilm-covered area relative to the total field of view (n = 8 fields at 20x). No statistical differences were found for pairwise comparisons between biofilm coverage under exposure to C_{18}-HSL and in the no AHL control (t-test, α = 0.05).

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To confirm the effect of C_{18}-HSL on the strain ATCC 29212, biofilms grown either under exposure to this AHL and with no AHL exposure and stained with a combination of

SYTO 9 and PI were analysed by confocal microscopy (Figure 36). After 6 hours of incubation time, microcolonies could be observed in which most cells were membrane-intact (SYTO 9-positive), whereas observation of membrane-damaged cells (PI-positive) was limited (Figure 36A). This observation confirms previous results obtained with 6-hour biofilms of *E. faecalis*, which mainly displayed viable cells independently of the presence of AHLs. Measurements of biofilm-covered areas revealed that control biofilms extended over 48 ± 15% of the field of view (FoV), whereas biofilms exposed to C_{18}-HSL displayed a coverage of 59 ± 11% of the FoV (Figure 36B). While this increase in biofilm coverage was not statistically significant (*t*-test, $\alpha = 0.05$), the observation of the photomicrographs confirms the presence of a denser biofilm upon exposure to C_{18}-HSL. These results indicate that the increase in biofilm cells is less accentuated with C_{18}-HSL alone than upon exposure to the mix of long-chain AHLs previously investigated. This could be explained by a possible synergistic effect of the AHLs present in the mix. However, this hypothesis was not further investigated in the present study. Taken together, these results confirm previous observations on biofilm formation acceleration in the presence of long-chain AHLs, and in particular of C_{18}-HSL.

Compounds with antimicrobial activity acting at sub-minimum inhibitory concentrations (sub-MIC) have been shown to be able to either reduce or increase biofilm formation, depending on the bacterial taxa (Davies et al., 2006). Thus, the potential antimicrobial activity of AHLs against *E. faecalis* was tested by solid plate MIC assay (Figure 37). AHL concentration in sterile paper discs was at least 100-fold the concentration used in biofilm assays. The absence of inhibition halos surrounding the AHL discs indicated that none of the AHLs tested was capable of inhibiting *E. faecalis* ATCC 29212 growth. Hence, the observed effects on biofilm formation are not the consequence of a sub-MIC effect, further confirming that the biofilm increases observed in this study were due to AHL exposure and suggesting either the interaction of AHLs with *E. faecalis* cellular components.

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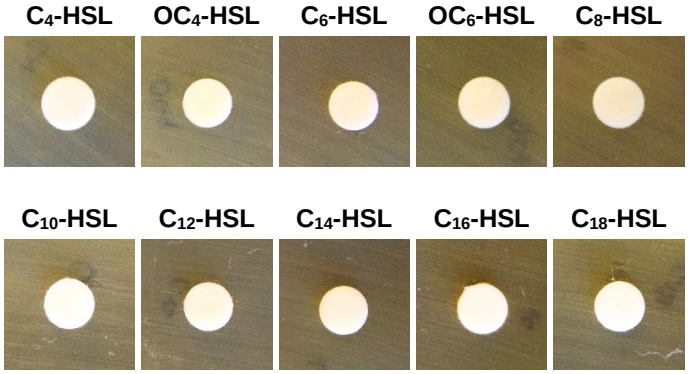


Figure 37. Minimum Inhibitory Concentration assay for AHLs on *E. faecalis* ATCC 29212. Overnight *E. faecalis* ATCC 29212 suspensions were plated on BHI, and discs bearing AHLs at concentrations 100-fold higher than the ones used in biofilm assays were placed on top of the agars. The homogenous growth surrounding the discs indicates no inhibitory activity of the AHLs assessed.

3.2. Influence of AHL integrity on E. faecalis biofilm formation

To determine whether the changes in *E. faecalis* biofilm generation were dependent on AHLs conserving their functional structure, biofilms were generated in the presence of degraded AHLs. Specifically, degradation was achieved by using either lactonase or acylase enzymes. Lactonases open the ring of AHLs, whereas acylases exscind the lactone ring from the acyl chain. The AHL C_{18}-HSL was chosen for these assays due to its significant effect on biofilm generation in *E. faecalis* ATCC 29212. To obtain the degraded AHL, C_{18}-HSL was diluted to the working concentration (1 μM) in fresh BHI and put in contact with either the lactonase Aii20J, acylase I from porcine kidney, or acylase I from *Aspergillus.* AHLs were incubated with the corresponding enzymes for 24 hours, and then, an aliquot of each condition was extracted, and C_{18}-HSL concentration was measured in all samples by HPLC-MS. The remaining volume was inoculated with overnight *E. faecalis* suspensions to generate biofilms. *E. faecalis* biofilms grown in BHI only and in BHI with each enzyme were used as no AHL negative controls.

Figure 38A displays the remaining concentration of C_{18}-HSL after degradation with each enzyme. Figure 38B displays the quantification by CV assay of *E. faecalis* ATCC 29212 biofilms exposed to C_{18}-HSL degraded by each enzyme. Biofilm mass in the no AHL control condition was not altered compared with biomass grown in the presence of the Aii20J lactonase (ANOVA, $\alpha = 0.05$). Contrarily, the addition of the acylase I enzyme, either of porcine kidney origin or purified from *Aspergillus*, induced significant increases compared with the no AHL-no enzyme control (ANOVA, $\alpha = 0.05$) (Figure 38B). The acylase I enzyme partially inactivates

AHLs, but is also active against several amino acids and amino acid derivates (Gentzen et al., 1980). Thus, the acylase enzyme may be able to cleave components of the BHI culture medium and causing a spurious increase in biofilm mass. Consequently, subsequent results obtained from the analysis of samples treated with the acylase enzymes have been assessed with caution.

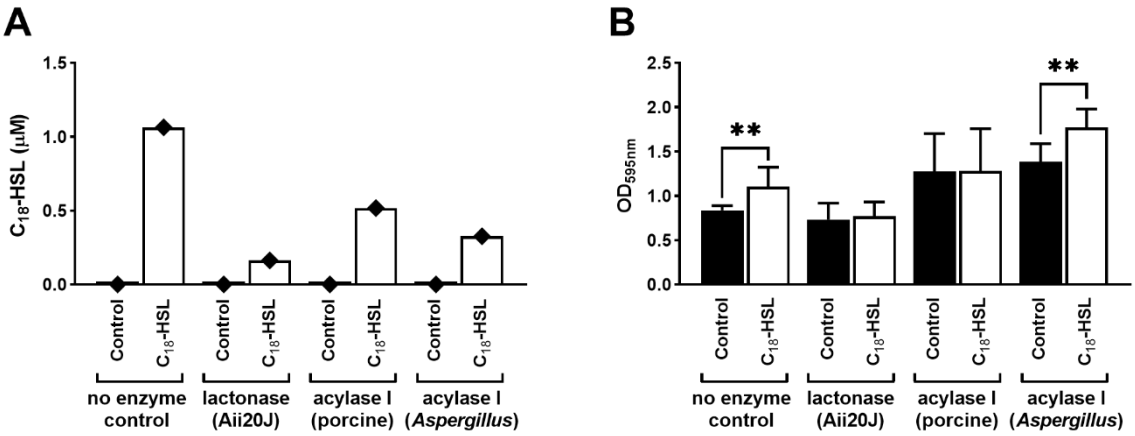


Figure 38. *E. faecalis* ATCC 29212 biofilm formation in the presence of C_{18}-HSL and its degradation products. **A.** Histograms represent the AHL concentration in no enzyme controls and after the incubation with Aii20J ("lactonase (Aii20J)"), acylase I from porcine kidney ("acylase I (porcine)"), and acylase I from *Aspergillus* ("acylase I (*Aspergillus*)"). The y-axis shows C_{18}-HSL concentration in micromolar, as assessed by HPLC-MS (n = 1). **B.** Histograms represent the amount of biofilm formed, as assessed by CV assays. The y-axis shows the amount of CV measured at OD_{595nm}. Bars display mean values of three independent experiments performed in triplicate, and whiskers represent SD (n = 9). Black bars represent biofilms that were not exposed to C_{18}-HSL, and white bars represent biofilms exposed to C_{18}-HSL at the concentrations shown in panel "A". Statistical significance of pairwise comparisons between each C_{18}-HSL condition and its no AHL control is displayed as **$p \leq 0.01$ (t-tests, $\alpha = 0.05$).

Once the effect of the addition of each enzyme on *E. faecalis* biofilm generation had been established, the effect of the degraded C_{18}-HSL was assessed. The addition of intact C_{18}-HSL significantly increased biofilm mass (Figure 38B), as seen in previous experiments in the same conditions (section 3.1 of this chapter). The degradation of C_{18}-HSL with the Aii20J lactonase reduced C_{18}-HSL to 16% of its initial concentration (Figure 38A). Exposure to C_{18}-HSL degraded by the lactonase did not result in biofilm mass increases compared with the no AHL control, nor with the no AHL-no enzyme control (t-test, $\alpha = 0.05$) (Figure 38B). This suggests that the lactone ring of the AHL must be intact for the increase in biofilm mass. Neither of the acylases tested reduced the levels of C_{18}-HSL as efficiently as the lactonase Aii20J, as detected by HPLC-MS (Figure 38A). These relatively low activity rates of acylase I against AHLs have already been reported for acylase I isolated from *Aspergillus* (Ivanova et al., 2015).

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In biofilms still exposed to 30 – 50% of the initial C_{18}-HSL concentration, quantified biofilm mass was still higher than in the no AHL-no enzyme control (Figure 38B). These observations could be due to the action of acylases on other compounds present in the culture medium.

3.3. Proteomic profiles in E. faecalis biofilms exposed to AHLs

The observation of significant increases in biofilm production under exposure to AHLs, specifically in *E. faecalis* ATCC 29212 under exposure to C_{18}-HSL, prompted the analysis of the proteomes of untreated biofilms and biofilms exposed to C_{18}-HSL. Proteins extracted from the biofilm samples were digested in solution and underwent label-free quantification. Protein identification was performed against the NCBI's protein database for *E. faecalis* ATCC 29212.

In the no AHL control condition, a total of 1068 proteins were identified and quantified, whereas a total of 855 proteins were identified and quantified in samples exposed to C_{18}-HSL (n = 3 replicates for each condition). The number of proteins identified was highly heterogenous between the replicates; therefore, only proteins that appeared in at least two of the three replicates were considered for subsequent analysis. This greatly reduced the number of proteins analysed. Among them, 57 proteins were common to both untreated biofilms and biofilms exposed to C_{18}-HSL (Figure 39), whereas 24 proteins were identified exclusively in no AHL control biofilms (Table 10), and 7 were unique to biofilms exposed to C_{18}-HSL (Table 11).

Figure 39 displays fold-change values of the number of spectra for each protein identified in the biofilms. Positive fold-change values indicate that the protein is more abundant in biofilms exposed to C_{18}-HSL than in no AHL controls, while negative fold-change values indicate the opposite. Fold-change values lower than -0.5 and higher than 0.5 were selected as thresholds for relevant differences in protein abundance. The majority of the proteins observed belong to central metabolic pathways. When using the number of spectra to calculate differential protein abundance, it is common that low-abundance proteins appear underrepresented (Lundgren et al., 2010). Hence, to investigate the proteins that appear at lower abundances, which normally constitute an important part of the proteome (Lundgren et al., 2010), a normalisation correction should be applied to these results.

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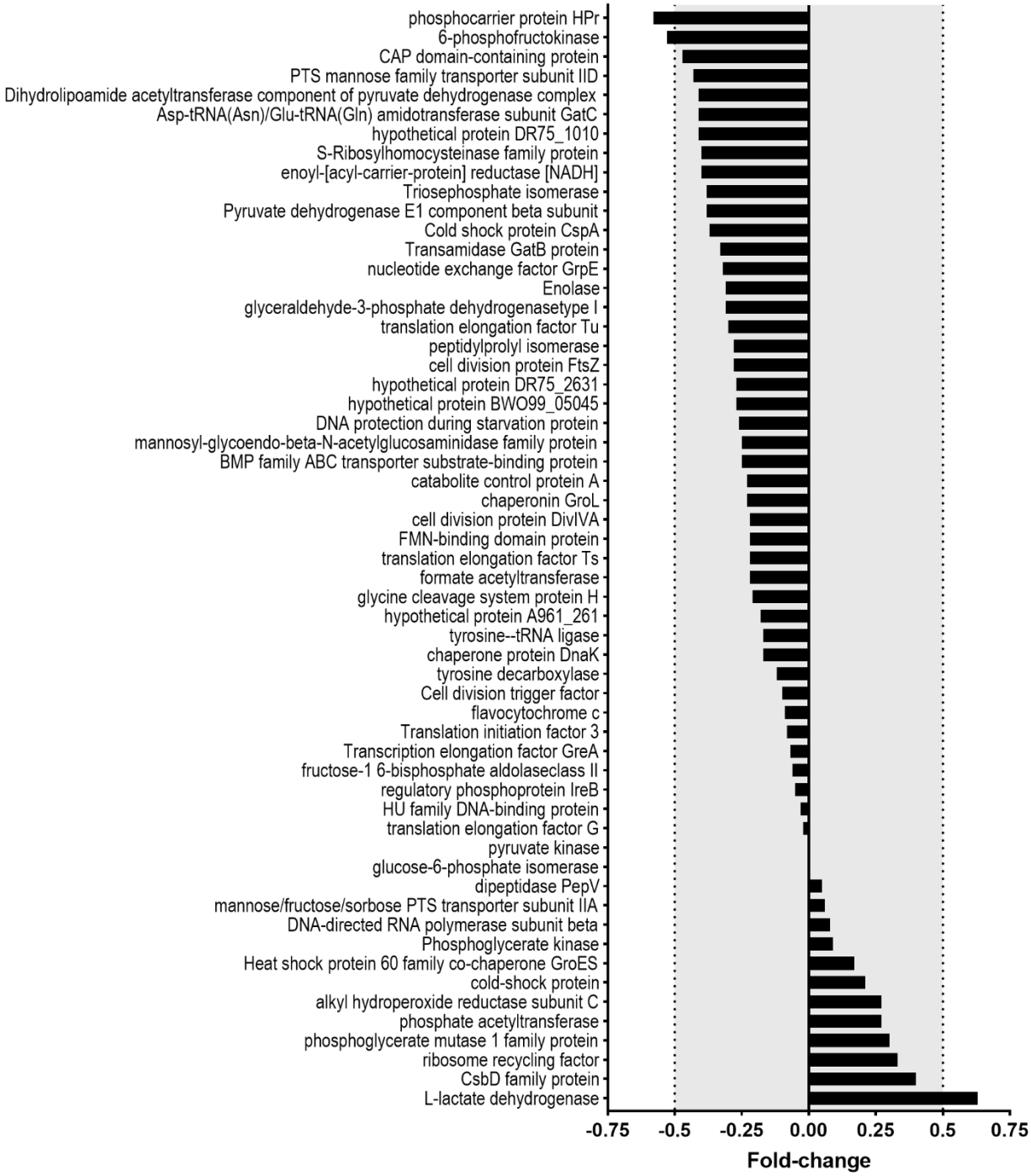


Figure 39. Differential expression of proteins in biofilms exposed to C_{18}-HSL compared with no AHL control biofilms. Horizontal bars show the relative protein expression on biofilms exposed to C_{18}-HSL, as fold-change values of the number of spectra identified for each protein (x-axis). Positive fold-change values indicate that the protein was overexpressed in biofilms exposed to C_{18}-HSL. The grey area marks the range of changes in protein levels not considered biologically relevant. The y-axis shows the protein description, as identified in the NCBI protein database for *E. faecalis* ATCC 29212 (NCBI Taxonomy ID: 1201292). Statistical comparisons between number of spectra assigned to each protein in no AHL controls and biofilms exposed to C_{18}-HSL were assessed with Mann-Whitney tests ($\alpha = 0.05$).

Statistical analysis of the differences in protein abundances revealed only two significantly down-regulated proteins in biofilms exposed to C_{18}-HSL: a phosphocarrier protein (HPr) and 6-phosphofructokinase (Figure 39). Both these proteins are involved in sugar metabolism, and thus, their down-regulation upon exposure to C_{18}-HSL seems to indicate a tendency of sugar metabolism shutdown. This response has been observed in oral polymicrobial biofilms upon fluoride treatment (López-López and Mira, 2020). Interestingly, among the proteins identified here, only one corresponded with the product of one of the transcripts investigated previously (see section 2.2. in this chapter). This protein was groL, corresponding to the product of the *groEL* transcript. As seen with the relative quantification of its transcript levels in strain ATCC 29212, the protein abundance was not significantly altered upon exposure to either long-chain AHLs or C_{18}-HSL (Figures 33 and 39).

Table 10. Proteins that were identified only in the no AHL control biofilms of *E. faecalis* ATCC 29212.

No.	Accession number	Unique peptides	Number of spectra	Description
1	gi|674289542	12	11	glutamine-fructose-6-phosphate transaminase
2	gi|401673840	7	8	GTP-binding protein TypA BipA
3	gi|1145684445	10	8	DNA-directed RNA polymerase subunit beta
4	gi|674290646	7	8	ATP-dependent metallopeptidase HflB family protein
5	gi|674289799	7	7	asparagine--tRNA ligase
6	gi|401672813	6	6	NADH dehydrogenase
7	gi|488285728	6	6	phosphoglucosamine mutase
8	gi|1145686198	6	6	protein translocase subunit SecA
9	gi|488284690	6	5	cell division protein FtsA
10	gi|1130381813	6	5	F0F1 ATP synthase subunit beta
11	gi|674290155	5	5	FMN-dependent NADH-azoreductase 2
12	gi|1145683852	6	5	phosphogluconate dehydrogenase
13	gi|488286557	4	4	dihydrolipoyl dehydrogenase
14	gi|1145687377	6	4	ATP synthase subunit alpha
15	gi|1145684800	5	4	NADH oxidase
16	gi|498517277	5	4	3-deoxy-7-phosphoheptulonate synthase
17	gi|1145683068	5	4	aspartyl/glutamyl-tRNA amidotransferase subunit B
18	gi|674289702	2	3	cold shock protein CspC
19	gi|401673745	4	3	RecA protein
20	gi|674289181	2	3	glyoxalase / Bleomycin resistance / Dioxygenase superfamily protein
21	gi|1145683192	3	3	2-dehydropantoate 2-reductase
22	gi|401674123	2	3	hypothetical protein A961_665
23	gi|401674742	2	2	CTP synthase
24	gi|488284763	2	2	redox-regulated ATPase YchF

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Table 11. Proteins that were identified only in *E. faecalis* ATCC 29212 biofilms exposed to C_{18}-HSL.

No.	Accession number	Unique peptides	Number of spectra	Description
1	gi|674288920	11	12	phosphoenolpyruvate-protein phosphotransferase
2	gi|488294232	7	9	NAD(P)/FAD-dependent oxidoreductase
3	gi|674290457	6	7	alanine--tRNA ligase
4	gi|401672750	3	4	hypothetical protein A961_2010
5	gi|488307973	3	4	cell division regulator GpsB
6	gi|401674078	2	3	hypothetical protein A961_692
7	gi|674288533	4	3	pyruvate dehydrogenase (acetyl-transferring) E1 component alpha subunit

Table 10 shows the proteins that were only identified in the no AHL control biofilms, whereas Table 11 shows the proteins that were only identified in biofilms exposed to C_{18}-HSL. To determine whether any of the proteins identified could explain the interactions of C_{18}-HSL with *E. faecalis* biofilms, those that appeared exclusively under exposure to C_{18}-HSL were further investigated. Among them, two hypothetical proteins appeared: A961_2010 and A961_692. Identification of these proteins in the UniProt database yielded a predicted annotation for A961_2010 as a CYTH domain-containing protein of only 63 amino acids. The second hypothetical protein, A961_692, was annotated as a probable transcriptional regulatory protein with a predicted molecular function of DNA binding. Further identification with the DAVID software yielded a predicted annotation as a YebC/PmpR family DNA-binding transcriptional regulator (accession number: WMS_RS15765). The YebC/PmpR family is conserved throughout different bacterial taxa (Zhang et al., 2012), with protein belonging to this family even linked to QS regulation in *P. aeruginosa* (Liang et al., 2008). Still, no experimental evidence on the function of these proteins has been described so far in enterococci. The identification of this protein exclusively in *E. faecalis* biofilms exposed to C_{18}-HSL opens the door to further studies on its implication in the regulation of the biofilm phenotype in the presence of AHLs.

Altogether, these results describe an effect of C_{18}-HSL on the induction of biofilm formation in *E. faecalis* strain ATCC 29212. The investigation of the proteome of *E. faecalis* biofilms exposed to C_{18}-HSL revealed a limited amount of proteins, but nevertheless, the presence of a yet-undescribed hypothetical transcriptional regulator was observed uniquely under exposure to the AHL. These results point towards a previously undescribed role of AHLs

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in *E. faecalis* biofilm formation, and they open the door to investigations on a hypothetical protein as a player behind these observations.

In this work, we have investigated the response of *E. faecalis* biofilms to the presence of AHLs, QS signals typically used by gram-negative bacteria. *E. faecalis* biofilms were characterised in terms of biomass, transcriptional regulations, and proteomic profiles in response to AHL exposure. Our findings demonstrate that *E. faecalis* strains of different origins respond to the addition of exogenous AHLs by significantly increasing their attaching biomass. Within the limits of this study, we describe biologically relevant up-regulation of QS-, biofilm- and stress-related transcripts upon AHL exposure, which could contribute to the expression of more virulent *E. faecalis* phenotypes. These regulations were highly dependent on the strain, highlighting the complexity of studying *E. faecalis* phenotypes. In addition, we observe predicted regulatory proteins that appear uniquely on biofilms exposed to AHLs, which function is yet-undescribed in enterococci. To the best of our knowledge, this is the first study that describes the differential regulation of virulence-related transcripts and proteins in *E. faecalis* in response to AHLs. Taken together, these results shed some light on intergeneric signalling pathways that are only beginning to be studied and open the door to further investigations on the underlying mechanisms that govern them. A deeper understanding of relationships between gram-positive and gram-negative bacteria is proposed as key in effectively tackling complex infections.

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Conclusions

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Conclusions

1. We describe that the use of subgingival samples as inocula, together with McBain culture medium and an anaerobic atmosphere in the Amsterdam Active Attachment model (AAA model) drives the generation of biofilms containing a representative proportion of gram-negative anaerobic or facultatively anaerobic taxa (33 – 43% total bacterial abundance), being the most suitable conditions to obtain periodontal microcosm biofilm models. When using other culture media to grow the same inocula, e.g., subgingival samples grown in BHI medium in an anaerobic atmosphere, the relative abundance of gram-negative bacteria in the *in vitro* biofilms was lower than 2%.
2. Best results in detecting biofilm sensitivity to the treatment with Aii20J have been obtained using the AAA model compared with the xCELLigence biofilm monitoring system, with the first providing numerous possibilities to study biofilm formation, biofilm microbiome structure and to perform imaging analyses. In addition, we observed subgingival biofilms to be more sensitive to the action of Aii20J, with 29 – 41% of samples significantly reduced, compared with saliva biofilms, with 24 – 29% of samples significantly reduced.
3. The use of Aii20J significantly reduces *in vitro* biofilm formation mechanisms within biofilms of oral origin, especially in samples from patients with oral diseases. Between 35 – 71% of periodontitis-derived biofilms presented macroscopical differences in the presence of Aii20J, presenting either a reduced biofilm density or less homogenous aspect. Microscopy analysis of biofilms from two subjects revealed lower biofilm-covered areas in Aii20J-treated biofilms, and shifts towards a higher presence of membrane-damaged cells in the presence of the enzyme, suggesting a different maturation among untreated and Aii20J-treated biofilms. These results were observed even in gram-positive dominated biofilms, with 40 – 60% of caries-derived biofilms exhibiting macroscopical differences due to the presence of Aii20J. Changes in biofilm mass and structure were derived from the enzymatic activity of Aii20J, since an inactivated Aii20J did not alter the biofilms. Altogether, these observations support the translation of Aii20J to the oral care field to be tested in *in vivo* trials.
4. AHLs or AHL-like molecules seem to play an important role in oral biofilm formation. This affirmation is supported by the high prevalence of sequences related to AHL synthases and promiscuous AHL receptors, especially of SdiA-related sequences, found

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in oral metagenomes from supragingival plaque samples. Besides, the significant biofilm reductions and structural changes observed under exposure to Aii20J, both in microbially diverse biofilms and gram-positive dominated biofilms, further reinforce this hypothesis and suggest the existence of a receptor in gram-positive taxa able to respond to AHLs or AHL-like molecules.

5. Despite the lack of success in detecting AHL production in *in vitro* biofilms by HPLC-MS, we cannot fully disregard the possibility of their production at low concentrations in the biofilm microhabitat. HPLC-MS spectra should be examined for non-standard AHLs and molecules structurally related to AHLs.
6. QQ processes against AHLs are present in high prevalence in saliva samples, further strengthening the hypothesis of the role of these molecules in the oral environment. In addition, protein sequences related to QQ enzymes appear to be extremely abundant among metagenomes of supragingival plaque samples. Investigation of the metagenomes of samples from different oral niches will confirm the widespread presence of these activities within the oral cavity.
7. Different *E. faecalis* strains respond to long-chain AHLs by specifically increasing their biofilm mass and microcolony formation.
8. *E. faecalis* exhibits biologically relevant differences in transcript levels of a selection of genes involved in AIP-mediated QS, biofilm formation, and membrane stress responses upon AHL exposure in a strain-dependent manner. The use of several strains of *E. faecalis* allows for accounting for differences in their genetic background and reduces biased conclusions regarding the effect of AHLs in this taxon.
9. AHLs could induce virulent phenotypes in some *E. faecalis* strains by up-regulating fsr-mediated QS responses. This observation would constitute the second report in the literature describing the cross-reactivity of AHLs and *agr* or *agr*-like QS systems in gram-positive taxa.
10. We hypothesise that the expression of a wider array of genes involved in biofilm formation may be altered in *E. faecalis* upon AHL exposure, given that differences in transcript levels of the biofilm-associated transcripts assessed here do not correlate with the biofilm phenotypes observed *in vitro*.
11. *E. faecalis* does not modify its central metabolism upon AHL exposure, as assessed by proteomic quantification using the number of spectra. These results indicate the need

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for a proteomic quantification approach that allows discerning changes in proteins present in very low abundance to further evaluate these results.

12. A hypothetical YebC/PmpR family DNA-binding transcriptional regulator protein with yet-undescribed function in enterococci was found only in *E. faecalis* biofilms exposed to the long-chain AHL C_{18}-HSL, opening the door to further studies on its implication in the regulation of the responses observed for the biofilm phenotype.
13. Altogether, these results point to a role of AHLs in a regulatory network among bacterial taxa that is more complex than contemplated in the current paradigm of cell-cell communication in the oral environment. Hence, it is necessary to continue studying the mechanisms underlying these processes in a more detailed manner, due to the therapeutic potential of new therapies based on AHL-targeting for oral biofilm modulation.

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Abstract

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Abstract

In recent years, oral health has gained importance as a factor contributing to general health and quality of life. In the global oral health status report of the WHO, the prevalence of the major oral diseases is shown for the year 2019, in which 2000 million people worldwide had untreated caries of permanent teeth, and 1000 million people suffered severe periodontal disease (World Health Organization, 2022). Due to the important role of bacteria in the onset and progression of these diseases, their control largely relies on the use of antimicrobials. The observed reduction in the efficacy of these treatments prompts the search for alternative, more targeted strategies to complement current oral disease management approaches.

In the mouth, bacteria are either present in a transient manner or embedded in a matrix of extracellular DNA and other polymers produced by themselves or by the host (Marsh, 2006). Bacteria within biofilms display distinct phenotypes compared with their planktonic counterparts and account for clinically relevant characteristics, such as an increased tolerance for antimicrobial treatments (Gilbert et al., 1997; Donlan and Costerton, 2002). In biofilms, an ecological equilibrium is maintained through competitive and cooperative microbial interactions (Marsh, 1994, 2003). Thus, changes in environmental conditions and the subsequent alteration of the equilibrium within the healthy oral biofilm have been intimately linked with the progression of the main oral diseases, dental caries and periodontitis (Marsh, 1994, 2003; Belda-Ferre et al., 2012; Hajishengallis et al., 2012). Hence, the modulation of the microbial interactions within the oral biofilm has been suggested as a strategy to regulate biofilm formation and ultimately control oral disease progression (Simón-Soro and Mira, 2015).

Cell density-dependent processes, termed quorum sensing (QS), have been closely related to biofilm formation. Moreover, interfering with QS processes, a strategy known as quorum quenching (QQ), has been proposed as a potential alternative for developing strategies to modulate oral biofilms (Cvitkovitch et al., 2003; Qi et al., 2005; Novak et al., 2010; Shao and Demuth, 2010; Jang et al., 2013; Muras et al., 2018, 2020b; Muras and Otero, 2020). Among the three most studied QS signals, the presence of gram-positive's autoinducer peptides (AIPs) and the universal autoinducer-2 (AI-2) have been described in oral bacteria (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Kolenbrander et al., 2006). Yet, the role of gram-negative's signalling molecules, the *N*-acyl-homoserine lactones (AHLs), has been traditionally disregarded in the context of oral disease progression, as AHL production was not detected in pure cultures of oral pathogens (Whittaker et al., 1996; Frias et al., 2001; Burgess

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et al., 2002; Jakubovics and Kolenbrander, 2010; Huang et al., 2011b; Guo et al., 2014). Despite this, several pieces of evidence have been found regarding AHL presence in oral samples (Kumari et al., 2006; Muras et al., 2020b) and AHL production by bacteria isolated from the mouth (Yin et al., 2012b, 2012a; Chen et al., 2013; Goh et al., 2014, 2016). Moreover, AHLs and analogues of AHLs can alter protein expression in *P. gingivalis* (Komiya-Ito et al., 2006; Asahi et al., 2010) and AHL production has been recently described in pure cultures of this periodontal pathogen (Muras et al., 2020b). Recently, AHL addition was described to affect the phenotypic activities of *in vitro* oral biofilms and to shift their microbial composition towards a periodontal bacterial profile (Muras et al., 2020a). In addition, gene clusters predicted to be related to homoserine lactone synthesis have been described in the bacterial metagenomes found in samples from caries and periodontitis patients (Aleti et al., 2019). Altogether, this evidence suggests that the role of AHLs in the oral cavity may be more important than previously thought and prompt the study of strategies that target these molecules as an alternative to modulate oral biofilm formation.

In the present thesis, we first aimed to evaluate the use of an AHL-targeting enzyme (Aii20J) as a biofilm modulatory strategy using *in vitro* biofilms derived from oral samples of either healthy or diseased origin. This strategy has been described to be effective in a previous study using saliva-derived biofilms (Muras et al., 2020b). Here, to obtain *in vitro* polymicrobial biofilms representative of the periodontal pocket microbiota, biofilms were inoculated with gingival crevicular fluid (GCF) from the subgingival pocket, obtained with paper points, and the results were compared with those obtained using saliva samples from the same donor. Biofilms were grown and quantified using two different biofilm models: the Amsterdam Active Attachment model (AAA model) (Exterkate et al., 2010; Muras et al., 2020a) together with crystal violet (CV assay, and the xCELLigence RTCA system (ACEA Biosciences Inc.) (Mira et al., 2019), which measures changes in impedance as cells attach to the bottom of the wells of the E-plates (ACEA Biosciences Inc.). In addition, biofilms were generated in different culture media and conditions. The initial culture media of choice were BHI and Schaedler supplemented with vitamin K1, two media rich in proteins. Subsequent experiments were done using semi-defined McBain medium (McBain et al., 2005), containing a much higher load of protein components, and a buffering agent that maintained the pH at 7. Biofilms were generated in either an aerobic or anaerobic atmosphere. We describe significant reductions in up to 29% of the saliva-derived biofilms and 41% of subgingival-derived biofilms grown in the AAA

model. Structural differences among untreated controls and Aii20J-treated biofilms were observed in up to 53% of saliva-derived biofilms and 65% of subgingival-derived biofilms, indicating that subgingival samples were more sensitive to the addition of Aii20J than saliva samples. Biofilms generated using different sample types from the same patient sometimes presented different responses to the addition of Aii20J, even though the biofilms obtained were, in either case, dominated by gram-positive taxa. This different responses to the Aii20J-treatment could be explained by differences in the microbiota present in each sampling site and present in low abundance levels in the *in vitro* biofilms (Simón-Soro et al., 2013; Frias-Lopez and Duran-Pinedo, 2020). The xCELLigence system was less sensitive to changes in biofilm in the presence of the enzyme, with only 12% of saliva-derived biofilms significantly reduced in the presence of the enzyme Aii20J and no significant differences observed for subgingival-derived biofilms. Thus, we propose using the AAA model for further studies on the effect of QQ strategies, as it additionally allows straightforward harvesting biofilm mass to extract gDNA and perform 16S community profiling of the samples.

Further investigations on the microbiome structure of *in vitro* revealed that the use of McBain culture medium and an anaerobic atmosphere drove the generation of the most diverse biofilms, as assessed by Illumina sequencing of the 16S rRNA gene. In these biofilms, the relative abundance of gram-negative anaerobes and facultative anaerobes increased up to 43% of total bacterial abundance. In contrast, for biofilms generated from the same inocula in BHI and anaerobiosis, relative abundance of these taxa was lower than 2%. Hence, we confirmed that using McBain and anaerobiosis conditions adequately represented the periodontal pocket habitat *in vitro*, as suggested by Janus et al. (2015). Despite other culture conditions resulting in biofilms dominated by gram-positive taxa, the addition of the enzyme resulted in significant reductions in most of the samples, suggesting an important role of AHLs produced by a minority of gram-negative species within the biofilm, or yet-undescribed AHL-like molecules sensed by gram-positive taxa. Interestingly, in none of the cases, the microbiome structure of the Aii20J-treated biofilms significantly differed from that of the untreated control, as assessed with Principal Coordinate Analysis and beta diversity indexes. This outcome is expected with the use of QQ strategies, as they should not directly affect bacterial viability.

A greater number of patients was recruited to further confirm our previous results on biofilm reductions and the conservation of the microbiome structure. Biofilms generated in the AAA model from subgingival samples were greatly reduced by Aii20J, with significant

reductions in 61% of gram-positive dominated biofilms (grown in BHI) and 39% of periodontal-profile biofilms (grown in McBain). Structural differences were observed in the presence of the enzyme for 87% of gram-positive dominated biofilms and 35% of biofilms with higher bacterial diversity. We confirmed previous observations on the enzyme not altering the microbiome structure of the samples. The apparent lower abundance of some periodontal pathogens, such as *Porphyromonas* spp., in Aii20J-treated samples, revealed by Linear Discriminant Analysis, was not corroborated when performing a qPCR with primers specifically targeting two *Porphyromonas* species: *P. gingivalis* and *P. endodontalis*.

Although HPLC-MS analyses of the biofilm cultures did not reveal the production of AHLs in the biofilms, the AHL C_4-HSL appeared in the clean culture medium in two independent batches, which implies an exogenous exposition of the biofilms to this molecule. Despite these results, we cannot rule out the possibility of the presence of AHLs within the biofilms at concentrations lower than the detection limit of the HPLC-MS analysis performed. Similarly, we discard neither the possibility that under these particular culture conditions, AHL production may not be induced in these *in vitro* biofilms.

To investigate whether AHL metabolic pathways are present in oral bacteria, we performed *in silico* examinations of shotgun oral metagenomes from supragingival plaque samples. The queries used to investigate these metagenomes comprised sequences of AHL synthases, AHL receptors, and AHL-degrading enzymes, all available in the NCBI's databases and with empirically demonstrated activity. This search revealed a surprisingly high prevalence of a family of sequences related to the AHL synthase HdtS, which has been described to produce at least three different AHLs, some of which differ from the structure of the most standard AHLs (Laue et al., 2000). Moreover, we observed the relatively high prevalence of a family of promiscuous AHL receptors, which have been described to also respond to molecules other than AHLs (Styles and Blackwell, 2018). Altogether, these results suggest that either AHLs or AHL-like molecules have a greater role in oral bacteria than currently accepted.

The interesting results on the effect of Aii20J reducing gram-positive dominated biofilms prompted us to study a classical gram-positive driven disease, dental caries. Due to the important prevalence of caries in deciduous teeth among children, we recruited caries-free and caries children to perform supragingival plaque samplings and generate gram-positive dominated biofilms *in vitro*. The initial microbiome structure of the samples used to generate the biofilms was also investigated by 16S community profiling, observing an overall

homogenous distribution of bacterial genera identified, with no significant differences at genus level between samples from caries-free children and children with caries. This observation aligns with the current understanding of oral diseases, which gives greater importance to the functional activities within the oral biofilms than the microbial composition *per se*. The microbial diversity of the initial samples was drastically reduced upon generation of *in vitro* biofilms due to the selection of streptococci, achieved through the use of McBain medium supplemented with sucrose and aerobic conditions. In line with the first experiments of the present thesis, Aii20J was able to significantly reduce gram-positive dominated biofilms in 20 – 40% of samples. Structural differences respect to untreated biofilms were observed for 40 – 60% of the supragingival-derived biofilms, with less dense and less homogenous biofilms observed upon Aii20J treatment. Sequencing of the 16S rRNA gene in these samples revealed that the microbiome structure was not altered by the presence of the enzyme, which was previously described in this thesis. In addition, when examining the distribution of the different OTUs belonging to the genus *Streptococcus*, we observed that one of the OTUs was significantly decreased in several Aii20J-treated biofilms that had reduced their biofilm mass in response to the enzyme, while a different streptococcal OTU significantly increased in the same samples. This substitution between such closely related taxa upon exposure to the QQ enzyme Aii20J seems to suggest the presence of a promiscuous receptor that could recognise AHLs or AHL-like molecules produced by taxa present at very low relative abundances within the biofilms. Altogether, these results further support the use of Aii20J as a biofilm-modulatory strategy and an alternative to antimicrobials for oral diseases besides periodontitis.

The evidence found in the first part of this thesis on QQ against AHLs affecting gram-positive dominated biofilms, together with scattered reports in the literature regarding the effect of AHLs on gram-positive bacteria in pure cultures (Qazi et al., 2006; Bensalem et al., 2014; Naik et al., 2017, 2018), and on multispecies biofilms containing gram-positive taxa (Janus et al., 2016; Muras et al., 2020a), encouraged the study of AHL addition to monospecies biofilms of a clinically relevant gram-positive pathogen; *E. faecalis*. The use of several *E. faecalis* strains allowed for accounting for this bacterium's intrinsic high genomic plasticity (Paulsen et al., 2003; Palmer et al., 2012), which can result in the observation of different responses towards the same stimuli. A selection of AHLs without inhibitory effects on *E. faecalis*, as assessed with a Minimum Inhibitory Concentration assay, was used for these experiments. The effect of AHL exposure was assessed at an early biofilm formation stage (6 hours of incubation), where

microcolonies could be observed under the confocal microscope. Exposure to a mix of long-chain AHLs (bearing between 10 and 18 carbon atoms in their acyl chains) resulted in significant increases of 33 to 42% of biofilm mass in several strains, as assessed with CV assay, with no effect on total planktonic growth. The response to both the short- and long-chain AHL mixes of one of the *E. faecalis* strains seems to indicate the potential presence of a promiscuous receptor that is sensing these molecules, probably in a secondary fashion respect to its cognate ligand. The response of *E. faecalis* to gram-negative signalling molecules could be relevant in a multispecies biofilm, where *E. faecalis* would be in close contact with bacteria able to produce these molecules.

The investigation, using RT-qPCR and relative quantification (ΔΔCt method), of a selection of transcripts related to peptide-mediated QS, biofilm formation and membrane stress responses revealed several relevant regulations that appeared in a strain-dependent manner. Relevant up-regulations (higher than 2-fold) of QS-related transcripts in strains ATCC 29212 and UmID7 suggest an increased virulence in these strains in the presence of AHLs. The regulations observed for the selection of genes associated with biofilm formation did not correlate with the phenotype observed when assessing biofilm mass in the same strains. This observation points to the possibility of the network of genes regulated upon AHL exposure being wider than what was assessed with the qPCR. The use of complementary, open-ended approaches would be useful to gain a deeper understanding of the changes occurring within the biofilm in the presence of the AHLs. Membrane stress response genes were only differentially regulated in one *E. faecalis* strain, UmID7, with relevant up-regulations that could also derive in the induction of more virulent phenotypes in the presence of especially long-chain AHLs.

In addition, *E. faecalis* strain ATCC 29212 was selected for further studies to elucidate the mechanisms behind the changes in biofilm behaviour in response to AHLs. First, *E. faecalis* biofilms were exposed to AHLs in an individual manner, observing that C_{18}-HSL was the sole AHL able to induce significant biofilm increases on its own, as assessed with CV assay. An increase in biofilm coverage upon exposure to C_{18}-HSL was also observed with confocal microscopy. The increase in biofilm was attributed the presence of C_{18}-HSL in its intact form, as exposure to a hydrolysed C_{18}-HSL, with its lactone ring open, resulted in the same phenotype as the no AHL control condition. This result suggests that the structure of the lactone ring may be necessary to induce the observed responses in this bacterium. To gain a deeper understanding of the changes undergoing the increase in biofilm in *E. faecalis*, biofilms were generated to

obtain their whole proteome, and shotgun proteomics were performed, using mass spectrometry analyses for protein identification. When examining the complete proteomic profiles of strain ATCC 29212 in the presence of C_{18}-HSL, the most abundant proteins were not altered, as assessed by comparing the number of spectra for each protein identified. These results suggest that it would be more suitable to choose a method of comparison that allowed a more precise assessment of proteins present at low abundance levels within the samples, as the number of spectra represents faithfully proteins present at high abundance levels. Nevertheless, two hypothetical proteins appeared exclusively in samples exposed to C_{18}-HSL: A961_2010 and A961_692. Identification of these proteins in the UniProt database and DAVID software yielded a predicted annotation for A961_692 as a probable transcriptional regulatory protein belonging to the YebC/PmpR family of DNA-binding transcriptional regulators. To date, no experimental evidence on the function of these proteins has been generated in *Enterococcus*. However, a protein belonging to this family has been described to regulate QS in *Pseudomonas aeruginosa* (Liang et al., 2008). These results open the door to further study of the responses of this putative transcriptional regulator to the presence of AHLs in other *E. faecalis* strains, as it was previously observed that they could present very different regulatory circuits. Altogether, these results point towards a previously undescribed role of AHLs in the regulation of *E. faecalis* biofilm formation.

The results obtained in the present thesis seem to indicate that the role of AHLs or AHL-like molecules in the oral environment has been underestimated. Targeting these molecules is presented as a promising approach to modulate biofilm formation *in vitro*, and further studies *in vivo* should be performed to validate this technology as a feasible alternative for antimicrobials in human oral health care. The confirmation of the antibiofilm effect of AHL disruption on biofilm communities dominated by gram-positive taxa led to the investigation of the interactions between AHLs and the gram-positive representative *E. faecalis*. The unequivocal induction of early biofilm formation in several *E. faecalis* strains in the presence of long-chain AHLs prompts the pursuit of additional investigations aiming to identify the molecular mechanisms behind these observed changes. Altogether, the results presented here contribute to the growing evidence of AHLs or AHL-like molecules having important roles within the oral environment, specifically within the oral biofilm.

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Resumen

En los últimos años, la importancia de la salud oral como factor que contribuye a la salud general y la calidad de vida ha ido en aumento. En el último informe acerca del estado de la salud oral global de la Organización Mundial de la Salud se recoge la prevalencia de las enfermedades orales más importantes en el año 2019. En ese año, 2000 millones de personas en todo el mundo sufrieron de caries sin tratar en dientes permanentes, y 1000 millones de personas padecieron formas graves de enfermedades periodontales (World Health Organization, 2022). Dada la importancia de las bacterias en el establecimiento y progresión de estas enfermedades, su control se basa en gran medida en el uso de antimicrobianos. La reducción de la eficacia de estos tratamientos ha promovido la búsqueda de alternativas más dirigidas que complementen las estrategias actuales para la gestión de las enfermedades orales.

En la boca, las bacterias pueden encontrarse de forma transitoria o incluidas en una matriz de ADN extracelular y otros polímeros producidos por ellas mismas o por el hospedador (Marsh, 2006). Las bacterias que habitan las biopelículas presentan fenotipos distintos a los de sus homólogas planctónicas y son responsables de características clínicamente relevantes, como una mayor tolerancia a los tratamientos antimicrobianos (Gilbert et al., 1997; Donlan y Costerton, 2002). En las biopelículas se mantiene un equilibrio ecológico mediante interacciones competitivas y cooperativas entre bacterias (Marsh, 1994, 2003). Así, cambios en las condiciones ambientales y la consiguiente alteración del equilibrio dentro de la biopelícula oral sana han sido estrechamente relacionados con la progresión de las principales enfermedades orales, la caries dental y la periodontitis (Marsh, 1994, 2003; Belda-Ferre et al., 2012; Hajishengallis et al., 2012). Por lo tanto, la modulación de las interacciones microbianas dentro de la biopelícula oral ha sido sugerida como una estrategia para regular la formación de biopelículas y, en última instancia, controlar la progresión de la enfermedad oral (Simón-Soro y Mira, 2015).

Los procesos dependientes de la densidad celular, denominados *quorum sensing* (QS), han sido estrechamente relacionados con la formación de biopelículas. Por tanto, interferir en los procesos de QS, estrategia conocida como *quorum quenching* (QQ), se ha propuesto como una potencial alternativa para el desarrollo de estrategias de modulación de las biopelículas orales (Cvitkovitch et al., 2003; Qi et al., 2005; Novak et al., 2010; Shao y Demuth, 2010; Jang et al., 2013; Muras et al., 2018, 2020b; Muras y Otero, 2020). De entre las tres señales de QS más estudiadas, la presencia de péptidos autoinductores (AIPs) de gram-positivas y del

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autoinductor universal-2 (AI-2) ha sido descrita en bacterias orales (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Kolenbrander et al., 2006). Sin embargo, el papel de las moléculas de señalización de las bacterias gram-negativas, las *N*-acil-homoserín lactonas (AHLs), ha sido tradicionalmente considerado no relevante en el contexto de la progresión de la enfermedad oral, ya que la producción de AHLs no se detectó en cultivos puros de patógenos orales en estudios clásicos (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Jakubovics y Kolenbrander, 2010; Huang et al., 2011b; Guo et al., 2014). A pesar de ello, se han encontrado varias pruebas de la presencia de AHLs en muestras orales (Kumari et al., 2006; Muras et al., 2020b) y de la producción de AHLs por bacterias aisladas de la boca (Yin et al., 2012b, 2012a; Chen et al., 2013; Goh et al., 2014, 2016). Además, las AHLs y análogos de estas moléculas han demostrado tener la capacidad de alterar la expresión de proteínas en *P. gingivalis* (Komiya-Ito et al., 2006; Asahi et al., 2010) y recientemente se ha descrito la producción de AHLs en cultivos puros de este periodontopatógeno (Muras et al., 2020b). En un estudio reciente, se describió cómo la adición de AHLs afecta a las actividades fenotípicas de biopelículas orales *in vitro* y hace cambiar su composición microbiana hacia un perfil bacteriano periodontal (Muras et al., 2020a). Además, se han descrito grupos de genes relacionados con la síntesis de homoserín lactonas en metagenomas bacterianos encontrados en muestras de pacientes con caries y periodontitis (Aleti et al., 2019). En conjunto, estas evidencias sugieren que el papel de las AHLs en la cavidad oral puede ser más importante de lo tradicionalmente establecido, e impulsan el estudio de estrategias dirigidas a estas moléculas como alternativas para modular la formación de la biopelícula oral.

En la presente tesis, en primer lugar se propuso la evaluación del uso de un enzima activo contra AHLs (Aii20J) como estrategia moduladora de la biopelícula, utilizando para ello biopelículas *in vitro* derivadas de muestras orales de origen sano o con enfermedades orales. Esta estrategia ha sido descrita como eficaz en un estudio anterior en el que se utilizaron biopelículas derivadas de saliva (Muras et al., 2020b). Aquí, para obtener biopelículas polimicrobianas *in vitro* que fueran representativas de la microbiota de la bolsa periodontal, se inocularon biopelículas a partir de fluido crevicular gingival (GCF) obtenido de la bolsa subgingival mediante la inserción de puntas de papel, y se compararon los resultados con los obtenidos utilizando muestras de saliva del mismo donante. Las biopelículas se cultivaron y cuantificaron utilizando dos modelos diferentes: una modificación del modelo de Adhesión Activa de Ámsterdam (modelo AAA, “Amsterdam Active Attachment model”) (Exterkate et

al., 2010; Muras et al., 2020a) junto con el ensayo de cristal violeta (CV), y el sistema xCELLigence RTCA (ACEA Biosciences Inc.) (Mira et al., 2019), que mide los cambios en la impedancia a medida que las células se adhieren al fondo de los pocillos de "E-plates" (ACEA Biosciences Inc.). Además, se generaron biopelículas en diferentes medios y condiciones de cultivo. Los medios de cultivo iniciales elegidos fueron BHI y Schaedler suplementado con vitamina K1, dos medios ricos en proteínas. Los experimentos posteriores se realizaron utilizando el medio semidefinido McBain (McBain et al., 2005), que contenía una carga mucho mayor de componentes proteínicos, y un agente tamponador que mantiene el pH en 7. Las biopelículas se generaron en una atmósfera aeróbica o anaeróbica. Describimos reducciones significativas de hasta el 29% de las biopelículas derivadas de muestras de saliva, y del 41% de las biopelículas subgingivales, cultivadas en el modelo AAA. Se observaron diferencias estructurales entre los controles sin tratamiento y las biopelículas tratadas con Aii20J en hasta el 53% de las biopelículas derivadas de la saliva y el 65% de las biopelículas derivadas de la zona subgingival. Estos resultados indican que las biopelículas derivadas de la zona subgingival son más sensibles a la actividad del enzima que las biopelículas derivadas de las muestras de saliva. En ocasiones, se observó que las biopelículas generadas a partir de muestras de un mismo paciente, diferían en comportamiento frente al enzima Aii20J según el tipo de muestra inicial empleada para la generación de las mismas. Esto ocurría incluso cuando las biopelículas obtenidas presentaban una composición microbiana similar, con una clara dominancia de representantes de bacterias gram-positivas. Estas diferencias en las respuestas al tratamiento con Aii20J podrían ser explicadas por las diferencias en la microbiota presente en cada uno de los nichos de los que se extraen las muestras iniciales, y que después pueden encontrarse presentes en baja abundancia en las biopelículas *in vitro* (Simón-Soro et al., 2013; Frias-Lopez and Duran-Pinedo, 2020). El sistema xCELLigence, por su parte, fue menos sensible a los cambios en la biopelícula en presencia del enzima, ya que solo el 12% de las biopelículas derivadas de saliva fueron significativamente reducidas en presencia de Aii20J, y no se observaron diferencias significativas para las biopelículas derivadas de la zona subgingival. Así pues, proponemos la utilización del modelo AAA para futuros estudios sobre el efecto de las estrategias QQ, ya que además permite recoger directamente la biomasa de las biopelículas para extraer ADN y realizar estudios acerca de la composición microbiana de las muestras.

El estudio de la estructura de los microbiomas obtenidos *in vitro* revelaron que el uso del medio de cultivo McBain junto con una atmósfera anaeróbica impulsaron la generación de

las biopelículas más diversas, según revelaron los resultados de la secuenciación del gen de ARNr 16S mediante Illumina. En estas biopelículas, la abundancia relativa de anaerobios gram-negativos y anaerobios facultativos aumentó hasta conformar el 43% de la abundancia bacteriana total. En cambio, en las biopelículas generadas a partir de esos mismos inóculos crecidos en BHI y anaerobiosis, la abundancia relativa de estos taxones fue inferior al 2%. Por lo tanto, confirmamos que el uso de las condiciones de McBain y anaerobiosis permite recrear adecuadamente el hábitat de la bolsa periodontal *in vitro*, como sugieren Janus y col. (2015). A pesar de que algunas condiciones de cultivo dieron lugar a biopelículas dominadas por bacterias gram-positivas, la adición del enzima dio lugar a reducciones significativas de biomasa en la mayoría de las muestras, lo que sugiere un papel importante de las AHLs producidas por una minoría de especies gram-negativas dentro de la biopelícula, o de moléculas similares a las AHLs, aún no descritas, que puedan ser detectadas por gram-positivas. Curiosamente, en ninguno de los casos la estructura del microbioma de las biopelículas tratadas con Aii20J difirió significativamente de la del control no tratado, según se evaluó mediante análisis de coordenadas principales (PCoA) e índices de diversidad beta. Este resultado es el esperado con el uso de estrategias de QQ, ya que no deberían afectar directamente a la viabilidad bacteriana.

Se reclutó un mayor número de pacientes para confirmar estos resultados acerca de la reducción de las biopelículas y la conservación de la estructura del microbioma de las mismas. Las biopelículas, generadas en el modelo AAA a partir de muestras subgingivales, se redujeron en gran medida en presencia de Aii20J, con reducciones significativas del 61% de las biopelículas dominadas por gram-positivas (cultivadas en BHI) y del 39% de las biopelículas de perfil periodontal (cultivadas en McBain). Se observaron diferencias estructurales en presencia del enzima en el 87% de las biopelículas dominadas por gram-positivas y en el 35% de las biopelículas con mayor diversidad bacteriana. Se confirmaron las observaciones anteriores acerca de la no alteración de la estructura del microbioma de las muestras en presencia del enzima. El análisis de discriminación lineal (LDA), reveló una aparente menor abundancia de algunos patógenos periodontales, como *Porphyromonas* spp., en las muestras tratadas con Aii20J, pero estas diferencias no fueron corroboradas al realizar una PCR cuantitativa empleando cebadores dirigidos específicamente a dos especies del género *Porphyromonas: P. gingivalis y P. endodontalis.*

Aunque los análisis por HPLC-MS de los cultivos de biopelículas orales *in vitro* no revelaron la producción de AHLs en dichas biopelículas, la AHL C_4-HSL apareció en el medio

de cultivo limpio en dos lotes de muestras independientes, lo que indica una exposición exógena de las biopelículas a esta molécula. A pesar de estos resultados, no podemos descartar la posibilidad de la presencia de AHLs dentro de las biopelículas en concentraciones inferiores al límite de detección del análisis HPLC-MS empleado. Del mismo modo, tampoco podemos descartar la posibilidad de que en estas condiciones de cultivo concretas no se induzca la producción de AHLs en estas biopelículas *in vitro*.

Para investigar si los principales elementos implicados en el metabolismo de AHLs están presentes en bacterias orales, se realizaron exámenes *in silico* de metagenomas orales de muestras de placa supragingival. Las secuencias utilizadas para investigar estos metagenomas comprendían sintasas de AHLs, receptores de AHLs, y enzimas degradadores de AHLs, todas ellas disponibles en las bases de datos del NCBI y cuya actividad había sido empíricamente demostrada. Esta búsqueda reveló una prevalencia sorprendentemente alta de una familia de secuencias relacionadas con la AHL sintasa HdtS, para la que se ha descrito la producción de al menos tres AHLs diferentes, algunas de las cuales difieren en estructura respecto a las AHLs más comunes (Laue et al., 2000). Además, se observó una prevalencia relativamente alta de una familia de receptores promiscuos de AHLs, que también responden a moléculas distintas de las AHLs (Styles y Blackwell, 2018). En conjunto, estos resultados sugieren que las AHLs, o moléculas similares a las AHLs, tienen un papel más importante en las bacterias orales de lo que está aceptado en el paradigma actual de la comunicación bacteriana en la cavidad oral.

Los interesantes resultados del efecto de Aii20J reduciendo biopelículas dominadas por gram-positivas impulsaron el estudio de una enfermedad oral cuya progresión está clásicamente ligada a bacterias gram-positivas: la caries dental. Debido a la importante prevalencia de caries de dientes deciduos en niños, se reclutaron niños con y sin caries para realizar muestreos de placa supragingival y generar biopelículas dominadas por bacterias gram-positivas *in vitro*. La estructura inicial del microbioma de las muestras utilizadas para generar las biopelículas se investigó la secuenciación del ARNr 16S, observando que los géneros bacterianos identificados más abundantes presentabas una distribución homogénea entre las muestras, sin diferencias significativas a nivel de género entre las muestras de niños sin caries y con caries. Esta observación concuerda con el enfoque actual de la progresión de las enfermedades orales, que da más importancia a las actividades funcionales dentro de las biopelículas orales que a la composición microbiana *per se*. La diversidad microbiana de las muestras iniciales se redujo drásticamente tras la generación de biopelículas *in vitro* debido a la selección de estreptococos,

lograda mediante el uso del medio McBain suplementado con sacarosa y la utilización de una atmósfera aeróbica. En línea con los primeros experimentos de esta tesis, Aii20J fue capaz de reducir significativamente las biopelículas dominadas por gram-positivas en el 20 – 40% de las muestras. Se observaron diferencias estructurales con respecto a las biopelículas no tratadas en el 40 – 60% de las biopelículas supragingivales, observándose biopelículas menos densas y menos homogéneas tras el tratamiento con Aii20J. La secuenciación del gen de ARNr 16S en estas muestras reveló que la estructura del microbioma no se veía alterada por la presencia del enzima, como se describió previamente en este trabajo. Además, al examinar la distribución de las diferentes unidades taxonómicas operativas (OTUs) pertenecientes al género *Streptococcus*, observamos que una de las OTUs estaba significativamente disminuida en varias biopelículas tratadas con Aii20J que habían respondido al enzima con una reducción de su biomasa, mientras que otra de las OTUs de *Streptococcus* aumentaba significativamente en las mismas muestras. Esta sustitución entre taxones tan estrechamente relacionados tras la exposición al enzima de QQ Aii20J parece sugerir la presencia de un receptor promiscuo que podría reconocer AHLs o moléculas similares a las AHLs producidas por taxones presentes en muy baja abundancia relativa dentro de las biopelículas. En conjunto, estos resultados apoyan el uso de Aii20J como estrategia para modular las biopelículas orales y como alternativa a los antimicrobianos para enfermedades orales distintas de la periodontitis.

Las pruebas encontradas en la primera parte de esta tesis acerca del potencial de estrategias QQ contra AHLs contra biopelículas dominadas por bacterias gram-positivas, junto con hallazgos dispersos en la literatura sobre el efecto de distintas AHLs en bacterias gram-positivas en cultivos puros (Qazi et al., 2006; Bensalem et al., 2014; Naik et al., 2017, 2018), y sobre biopelículas polimicrobianas que contienen taxones gram-positivos (Janus et al., 2016; Muras et al., 2020a), alentaron el estudio de la adición de AHLs a biopelículas monoespecie de un patógeno gram-positivo de relevancia clínica: *E. faecalis*. El uso de varias cepas de *E. faecalis* permitió tener en cuenta la alta plasticidad genómica intrínseca a esta especie (Paulsen et al., 2003; Palmer et al., 2012), que puede dar lugar a la observación de diferentes respuestas a nivel genómico y fenotípico ante los mismos estímulos. Para estos experimentos se utilizó una selección de AHLs que no presentaban efectos inhibitorios sobre *E. faecalis*, tal y como se evaluó mediante ensayos de Concentración Mínima Inhibitoria (MIC). El efecto de la exposición a AHLs se evaluó en una fase temprana de formación de biopelículas (6 horas de incubación), en la que podía observarse la formación de microcolonias al microscopio confocal.

La exposición a una mezcla de AHLs de cadena larga (con entre 10 y 18 átomos de carbono en sus cadenas de tipo acilo) produjo aumentos significativos del 33% al 42% de la biomasa de la biopelícula en varias cepas, según se evaluó con ensayos de tinción con CV, sin afectar, no obstante, al crecimiento planctónico total. La respuesta a las mezclas de AHLs de cadena corta y larga de una de las cepas de *E. faecalis* estudiada parece indicar la posible presencia de un receptor promiscuo que detecta estas moléculas, probablemente de manera secundaria respecto a su ligando nativo. La respuesta de *E. faecalis* a moléculas de señalización de bacterias gram-negativas podría ser relevante en biopelículas polimicrobianas, donde *E. faecalis* estaría en estrecho contacto con bacterias capaces de producir estas moléculas.

La investigación, mediante reverso-transcripción y PCR cuantitativa, empleando el método de cuantificación relativa (ΔΔCt), de una selección de transcritos relacionados con el QS mediada por péptidos, la formación de biopelículas y las respuestas al estrés de membrana reveló varias regulaciones relevantes asociadas a cada una de las cepas a estudio. En las cepas ATCC 29212 y UmID7 se observó un aumento biológicamente relevante (de más del doble) de los transcritos relacionados con el QS, lo que sugiere un aumento de la virulencia de estas cepas en presencia de AHLs. Las regulaciones observadas para la selección de genes asociados a la formación de biopelículas no se correlacionaron con el fenotipo observado al evaluar la biomasa en esas mismas cepas. Esta observación apunta a la posibilidad de que la red de genes regulados tras la exposición a AHLs sea más amplia de lo que se abarcó con el uso de la PCR cuantitativa. El uso de técnicas complementarias sería útil para lograr una mejor comprensión de los cambios que se producen en las biopelículas en presencia de las AHLs. Los genes de respuesta al estrés de membrana sólo se encontraron diferencialmente regulados en una de las cepas de *E. faecalis*, UmID7, con incrementos relevantes en sus niveles de expresión en presencia de AHLs, especialmente de cadena larga, que podrían derivar en la inducción de fenotipos más virulentos en esta cepa.

Posteriormente, se seleccionó la cepa *E. faecalis* ATCC 29212 para realizar nuevos estudios con el fin de dilucidar los mecanismos subyacentes a los cambios en el comportamiento de la biopelícula observados en respuesta a las AHLs. En primer lugar, biopelículas de *E. faecalis* ATCC 29212 fueron expuestas a AHLs de forma individual, observándose que C_{18}-HSL era la única AHL capaz de inducir por sí sola un aumento significativo de la biopelícula, según se evaluó con la tinción de CV. También se observó con microscopía confocal un aumento de la cobertura de la biopelícula tras la exposición a C_{18}-HSL. El aumento de la

biopelícula se atribuyó a la presencia de C_{18}-HSL en su forma intacta, ya que la exposición a una C_{18}-HSL hidrolizada, con su anillo lactona abierto, daba lugar al mismo fenotipo que la biopelícula control no expuesta a AHLs. Este resultado sugiere que la estructura del anillo lactona puede ser necesaria para inducir las respuestas observadas en esta bacteria. Para profundizar en el conocimiento de los cambios experimentados durante el aumento de la biopelícula en *E. faecalis*, se generaron biopelículas de las cuales obtener el proteoma completo para realizar proteómica "shotgun", utilizando análisis de espectrometría de masas para la identificación de proteínas. Al examinar los perfiles de proteómica de la cepa ATCC 29212 en presencia de C_{18}-HSL, las proteínas más abundantes no se vieron alteradas, según se observó al comparar el número de espectros para cada proteína identificada en las muestras control, y en las muestras tratadas con C_{18}-HSL. Estos resultados sugieren que sería más adecuado elegir un método de comparación que permitiera una evaluación más precisa de las proteínas presentes en baja abundancia en las muestras, ya que el número de espectros representa fielmente las proteínas presentes en abundancia alta. No obstante, la aparición de dos proteínas hipotéticas fue descrita exclusivamente en las muestras expuestas a C_{18}-HSL: A961_2010 y A961_692. La identificación de estas proteínas en la base de datos UniProt y el software DAVID resultó en una anotación predicha para A961_692 como una proteína reguladora transcripcional perteneciente a la familia YebC/PmpR de reguladores transcripcionales de unión al ADN. Hasta la fecha, no se han obtenido pruebas empíricas de la función de estas proteínas en *Enterococcus*. Sin embargo, se ha descrito que una proteína perteneciente a esta familia regula el QS en *Pseudomonas aeruginosa* (Liang et al., 2008). Estos resultados abren la puerta a la continuación del estudio de las respuestas de este regulador transcripcional putativo a la presencia de AHLs en otras cepas de *E. faecalis*, ya que anteriormente se observó que podían presentar circuitos reguladores muy diferentes. En conjunto, estos resultados apuntan a un papel hasta ahora no descrito de las AHLs en la regulación de la formación de biopelículas de *E. faecalis*.

Los resultados obtenidos en la presente tesis parecen indicar que el papel de las AHLs o de moléculas similares a las AHLs ha sido infravalorado en el contexto de la microbiología oral. Estas moléculas se presentas como una diana prometedora para la modulación de la formación de biopelículas *in vitro*, y se propone la realización de estudios *in vivo* para validar esta tecnología como una alternativa factible a los antimicrobianos para el cuidado de la salud oral humana. La confirmación del efecto inhibitorio sobre biopelículas de la disrupción de AHLs en comunidades de biopelículas dominadas por taxones gram-positivos condujo a la

investigación de las interacciones entre AHLs y la bacteria gram-positiva *E. faecalis*. La inequívoca inducción de la formación de biopelícula temprana en varias cepas de *E. faecalis* en presencia de AHLs de cadena larga impulsó investigaciones adicionales destinadas a identificar los mecanismos moleculares que subyacían a estos cambios. En conjunto, los resultados aquí presentados contribuyen a la creciente evidencia de que las AHLs, o incluso moléculas similares a las AHLs, desempeñan importantes funciones en el entorno oral, concretamente en la biopelícula oral.

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Resumo

Nos últimos anos, a importancia da saúde bucodental como factor que contribúe á saúde xeral e á calidade de vida foi aumentando. O último informe sobre o estado da saúde bucodental global da Organización Mundial da Saúde inclúe a prevalencia das enfermidades bucodentais máis importantes en 2019. Nese ano, 2000 millóns de persoas en todo o mundo tiveron caries sen tratar en dentes permanentes e 1.000 millóns de persoas padeceron de formas de enfermidade periodontal grave (World Health Organization, 2022). Dada a importancia das bacterias no establecemento e progresión destas enfermidades, o seu control baséase en gran medida no uso de antimicrobianos. A redución da eficacia destes tratamentos impulsou a busca de alternativas máis dirixidas que complementen as estratexias actuais para o manexo das enfermidades bucodentais.

Na boca, as bacterias pódense atopar de forma transitoria ou incluídas nunha matriz de ADN extracelular e outros polímeros producidos por eles mesmos ou polo hóspede (Marsh, 2006). As bacterias que habitan en biofilmes teñen fenotipos diferentes das súas homólogas planctónicas e son responsables de características clinicamente relevantes, como unha maior tolerancia aos tratamentos antimicrobianos (Gilbert et al., 1997; Donlan e Costerton, 2002). Nos biofilmes mantense un equilibrio ecolóxico mediante interaccións competitivas e cooperativas entre bacterias (Marsh, 1994, 2003). Así, os cambios nas condicións ambientais e a consecuente alteración do equilibrio dentro do biofilme oral saudable foron estreitamente relacionados coa progresión das principais enfermidades bucodentais: a carie dental e a periodontite (Marsh, 1994, 2003; Belda-Ferre et al., 2012; Hajishengallis et al., 2012). Polo tanto, a modulación das interaccións microbianas dentro do biofilm oral foi suxerida como unha estratexia para regular a formación de biofilm e, finalmente, controlar a progresión da enfermidade bucal (Simón-Soro e Mira, 2015).

Os procesos dependentes da densidade celular, coñecidos como *quorum sensing* (QS), están estreitamente relacionados coa formación de biofilmes bacterianos. Polo tanto, interferir cos procesos de QS, unha estratexia coñecida como *quorum quenching* (QQ), propúxose como unha potencial alternativa para o desenvolvemento de estratexias de modulación de biofilmes orais (Cvitkovitch et al., 2003; Qi et al., 2005; Novak et al., 2005; Novak et al. al., 2010; Shao e Demuth, 2010; Jang et al., 2013; Muras et al., 2018, 2020b; Muras e Otero, 2020). De entre os tres sinais de QS mellor estudados, a presenza de péptidos autoindutores (AIPs) de gram-positivas e o autoindutor universal-2 (AI-2) describiuse en bacterias orais (Whittaker et al.,

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1996; Frias et al., 2001). ; Burgess et al., 2002; Kolenbrander et al., 2006). Non obstante, o papel das moléculas de sinalización das bacterias gram-negativas, as *N*-acil-homoserín lactonas (AHLs), tradicionalmente foi considerado non relevante no contexto da progresión das enfermidades orais, xa que a produción de AHLs non fora detectada en cultivos puros de patóxenos orais (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Jakubovics e Kolenbrander, 2010; Huang et al., 2011b; Guo et al., 2014). A pesar diso, atopáronse varias evidencias da presenza de AHLs en mostras orais (Kumari et al., 2006; Muras et al., 2020b) e da produción de AHLs por bacterias illadas da boca (Yin et al., 2012b, 2012a; Chen et al., 2013; Goh et al., 2014, 2016). Ademais, demostrouse que as AHLs e análogos destas moléculas teñen a capacidade de alterar a expresión das proteínas en *P. gingivalis* (Komiya-Ito et al., 2006; Asahi et al., 2010) e recentemente describiuse a produción de AHLs en cultivos puros deste periodontopatóxeno (Muras et al., 2020b). Nun estudo recente, describiuse como a adición de AHLs afecta ás actividades fenotípicas dos biofilmes orais *in vitro* e cambia a súa composición microbiana cara un perfil bacteriano periodontal (Muras et al., 2020a). Ademais, describíronse grupos de xenes relacionados coa síntese de homoserín lactonas en metaxenomas bacterianos de mostras de pacientes con carie e periodontite (Aleti et al., 2019). En conxunto, estas evidencias suxiren que o papel das AHLs na cavidade oral pode ser máis importante que o tradicionalmente establecido, e fomentan o estudo de estratexias dirixidas a estas moléculas como alternativas para modular a formación de biofilmes orais.

Na presente tese propúxose, en primeiro lugar, a avaliación do uso dun encima activo fronte ás AHLs (Aii20J) como estratexia moduladora de biofilme, utilizando biofilmes *in vitro* derivados de mostras orais de orixe sa ou con enfermidades orais. Esta estratexia foi descrita como eficaz nun estudo previo que utilizou biofilmes derivados da saliva (Muras et al., 2020b). Aquí, para obter biofilmes polimicrobianos *in vitro* que fosen representativos da microbiota do peto periodontal, inoculáronse biofilmes a partir do líquido crevicular xenxival (GCF) obtido do peto subxenxival mediante a inserción de puntos de papel, e os resultados comparáronse cos obtidos mediante mostras de saliva do mesmo doador. Os biofilmes foron cultivados e cuantificados utilizando dous modelos diferentes: unha modificación do modelo de Adhesión Activa de Ámsterdam (modelo AAA, "Amsterdam Active Attachment model") (Exterkate et al., 2010; Muras et al., 2020a) xunto con tinguiduras con cristal violeta (CV), e o sistema xCELLigence RTCA (ACEA Biosciences Inc.) (Mira et al., 2019), que mide os cambios na impedancia a medida que as células se adhiren ao fondo das "E-plates" (ACEA Biosciences

Inc.). Ademais, xeráronse biofilmes en diferentes medios e condicións de cultivo. Os medios de cultivo iniciais elixidos foron BHI e Schaedler suplementado con vitamina K1, dous medios ricos en proteínas. Os experimentos posteriores realizáronse utilizando medio semidefinido McBain (McBain et al., 2005), que contén unha carga moito maior de compoñentes proteicos, e un axente tamponador que mantén o pH en 7. Os biofilmes xeráronse nunha atmosfera aerobia ou anaerobia. Describimos reducións significativas de ata o 29% dos biofilmes derivados de mostras de saliva, e do 41% dos biofilmes subxenxivais, cultivados no modelo AAA. Observáronse diferenzas estruturais entre os controis non tratados e os biofilmes tratados con Aii20J en ata o 53% dos biofilmes derivados de saliva e o 65% dos biofilmes subxenxivais. Estes resultados indican que os biofilmes derivados da área subxenxival son máis sensibles á actividade encimática de Aii20J que os biofilmes derivados de mostras de saliva. En ocasións, observouse que os biofilmes xerados a partir de mostras dun mesmo paciente diferían no seu comportamento fronte ao encima Aii20J dependendo do tipo de mostra inicial empregada para xeralos. Isto ocorreu mesmo cando os biofilmes obtidos tiñan unha composición microbiana similar, cun claro dominio de representantes de bacterias gram-positivas. Estas diferenzas nas respostas ao tratamento con Aii20J poderían explicarse polas diferenzas na microbiota presente en cada un dos nichos dos que se extraen as mostras iniciais, e que posteriormente poden atoparse presentes en pouca abundancia nos biofilmes *in vitro* (Simón-Soro et al., 2013; Frias-López e Duran-Pinedo, 2020). O sistema xCELLigence, pola súa banda, foi menos sensible aos cambios no biofilme en presenza do encima, xa que só un 12% dos biofilmes derivados da saliva foron significativamente reducidos en presenza de Aii20J, e non se observaron diferenzas significativas para os biofilmes derivados da zona subxenxival. Así, propoñemos o uso do modelo AAA para futuros estudos sobre o efecto das estratexias QQ, xa que permite tamén a recollida directa da biomasa dos biofilmes para extraer ADN e realizar estudos sobre a composición microbiana das mostras.

O estudo da estrutura dos microbiomas obtidos *in vitro* revelou que o uso do medio de cultivo McBain xunto cunha atmosfera anaerobia promove a xeración dos biofilmes máis diversos, tal e como revelan os resultados da secuenciación do xene de ARNr 16S mediante Illumina. Nestes biofilmes, a abundancia relativa de anaerobios gram-negativos e anaerobios facultativos aumentou ata constituír o 43% da abundancia total de bacterias. Pola contra, nos biofilmes xerados a partir deses mesmos inóculos cultivados en BHI e anaerobiose, a abundancia relativa destes taxons foi inferior ao 2%. Polo tanto, confirmamos que o uso de

McBain e condicións anaerobias permite recrear adecuadamente o hábitat do peto periodontal *in vitro*, como suxiren Janus e col. (2015). A pesar de que algunhas condicións de cultivo orixinaron biofilmes dominados por bacterias gram-positivas, a adición do encima levou a reducións significativas da biomasa na maioría das mostras, o que suxire un papel importante das AHL producidas por unha minoría de especies gram-negativas dentro do biofilme, ou de moléculas similares ás AHL, aínda non descritas, que poden ser detectadas por gram-positivos. Curiosamente, en ningún dos casos a estrutura do microbioma dos biofilmes tratadas con Aii20J difería significativamente da do control non tratado, segundo se avaliou mediante a análise de coordenadas principais (PCoA) e os índices de diversidade beta. Este resultado espérase co uso de estratexias QQ, xa que non deberían afectar directamente á viabilidade bacteriana.

Recrutouse un maior número de pacientes para confirmar estes resultados sobre a redución do biofilme e a preservación da estrutura do microbioma do mesmo. Os biofilmes, xerados no modelo AAA a partir de mostras subxenxivais, reducíronse en presenza de Aii20J, con reducións significativas do 61% dos biofilmes dominados por gram-positivos (cultivados en BHI) e do 39% dos biofilmes de perfil periodontal (cultivados en McBain). Observáronse diferenzas estruturais na presenza do encima no 87% dos biofilmes dominados por gram-positivos e no 35% dos biofilmes con maior diversidade bacteriana. Confirmáronse observacións previas sobre a non alteración da estrutura do microbioma das mostras en presenza do encima. A análise de discriminación lineal (LDA) revelou unha aparente menor abundancia dalgúns patóxenos periodontais, como *Porphyromonas* spp., nas mostras tratadas con Aii20J, pero estas diferenzas non foron corroboradas ao realizar unha PCR cuantitativa utilizando cebadores dirixidos especificamente a dúas especies do xénero *Porphyromonas: P. gingivalis e P. endodontalis.*

Aínda que a análise por HPLC-MS de cultivos de biofilmes orais *in vitro* non revelou a produción de AHLs en biofilmes orais, a AHL C_4-HSL apareceu en medio de cultivo limpo en dous lotes de mostras independentes, o que implica unha exposición esóxena dos biofilmes a esta molécula. A pesar destes resultados, non podemos descartar a posibilidade da presenza de AHLs dentro dos biofilmes a concentracións inferiores ao límite de detección da análise HPLC-MS empregada. Do mesmo xeito, non podemos descartar a posibilidade de que non se induza a produción de AHLs nestes biofilmes *in vitro* nestas condicións específicas de cultivo.

Para investigar se os principais elementos implicados no metabolismo das AHLs están presentes nas bacterias orais, realizáronse exames *in silico* de metaxenomas orais de mostras de

placas supraxenxivais. As secuencias utilizadas para investigar estes metaxenomas comprendían AHL sintases, receptores de AHLs e encimas que degradan AHLs, tódolos cales atópanse dispoñibles nas bases de datos do NCBI e cuxa actividade foi demostrada empiricamente. Esta busca revelou unha abundancia sorprendentemente alta dunha familia de secuencias relacionadas coa AHL sintase HdtS, para as que se describiu a produción de polo menos tres AHLs diferentes, algunhas das cales difiren en estrutura das AHL máis comúns (Laue et al. , 2000). Ademais, observouse unha abundancia relativamente alta dunha familia de receptores de AHLs promiscuos, que tamén responden a moléculas distintas das AHLs (Styles e Blackwell, 2018). En conxunto, estes resultados suxiren que as AHLs, ou moléculas similares a AHLs, teñen un papel máis importante nas bacterias orais do que se acepta no paradigma actual de comunicación bacteriana na cavidade oral.

Os interesantes resultados do efecto de Aii20J reducindo biofilmes dominados por gram-positivos motivaron o estudo dunha enfermidade bucal cuxa progresión está clasicamente ligada ás bacterias gram-positivas: a caries dental. Debido á significativa prevalencia de caries en dentes primarias nos nenos, recrutáronse nenos con e sen caries para tomar mostras de placa supraxenxival e xerar biofilmes dominados por bacterias gram-positivas *in vitro*. A estrutura inicial do microbioma das mostras utilizadas para xerar os biofilmes investigouse mediante secuenciación do ARNr 16S, observándose que os xéneros bacterianos máis abundantes identificados presentaban unha distribución homoxénea entre as mostras, sen diferenzas significativas a nivel de xénero entre as mostras dos nenos sen caries e con caries. Esta observación é consistente co enfoque actual da progresión da enfermidade oral, que dá máis importancia ás actividades funcionais dentro dos biofilms orais que á composición microbiana *per se*. A diversidade microbiana das mostras iniciais reduciuse drasticamente tras a xeración de biofilmes *in vitro* debido á selección de estreptococos, conseguida mediante o uso do medio de McBain complementado con sacarosa e o uso dunha atmosfera aerobia. En liña cos primeiros experimentos desta tese, Aii20J foi capaz de reducir significativamente os biofilmes dominados por gram-positivos no 20 – 40% das mostras. Observáronse diferenzas estruturais con respecto aos biofilmes non tratados no 40 – 60% dos biofilmes supraxenxivais, observándose biofilmes menos densos e menos homoxéneos despois do tratamento con Aii20J. A secuenciación do xene do ARNr 16S nestas mostras revelou que a estrutura do microbioma non se viu alterada pola presenza do encima, como se describiu anteriormente neste traballo. Ademais, ao examinar a distribución das diferentes unidades taxonómicas operativas (OTUs) pertencentes ao xénero

Streptococcus, observamos que unha das OTUs diminuía significativamente en varios biofilmes tratados con Aii20J que responderan ao encima cunha redución da súa biomasa, mentres que outra das OTUs de *Streptococcus* aumentaba significativamente nas mesmas mostras. Esta substitución entre taxons estreitamente relacionados tras a exposición ao encima Aii20J QQ parece suxerir a presenza dun receptor promiscuo que podería recoñecer AHLs ou moléculas similares a AHLs, producidas por taxons presentes en moi baixa abundancia relativa dentro dos biofilmes. En conxunto, estes resultados apoian o uso de Aii20J como estratexia para modular biofilmes orais e como alternativa aos antimicrobianos para enfermidades orais distintas da periodontite.

A evidencia atopada na primeira parte desta tese sobre o potencial das estratexias de QQ fronte ás AHL reducindo biofilmes dominados por bacterias gram-positivas, xunto con achados dispersos na literatura sobre o efecto de diferentes AHLs sobre bacterias gram-positivas en cultivo puro (Qazi et al., 2006; Bensalem et al., 2014; Naik et al., 2017, 2018) e sobre biofilmes polimicrobianos que conteñen taxons gram-positivos (Janus et al., 2016; Muras et al., 2020a), incentivaron o estudo da adición de AHLs a biofilmes monoespecies dun patóxeno gram-positivo de relevancia clínica: *E. faecalis.* O uso de varias cepas de *E. faecalis* permitiu ter en conta a alta plasticidade xenómica intrínseca a esta especie (Paulsen et al., 2003; Palmer et al., 2012), o que pode levar á observación de diferentes respostas a nivel xenómico e fenotípico fronte aos mesmos estímulos. Para estes experimentos, utilizouse unha selección de AHLs que non mostraron efectos inhibitorios sobre *E. faecalis*, segundo se avaliou mediante ensaios de concentración mínima inhibitoria (MIC). Avaliouse o efecto da exposición ás AHLs nunha fase inicial da formación de biofilmes (6 horas de incubación), na que se puido observar a formación de microcolonias ao microscopio confocal. A exposición a unha mestura de AHLs de cadea longa (con entre 10 e 18 átomos de carbono nas súas cadeas de acilo) produciu aumentos significativos do 33% ao 42% na biomasa de biofilme en varias cepas, segundo se avaliou mediante ensaios de tinción con CV, sen afectar, porén, ao crecemento planctónico total. A resposta ás mesturas de AHLs de cadea curta e longa dunha das cepas de *E. faecalis* estudadas parece indicar a posible presenza dun receptor promiscuo que detecte estas moléculas, probablemente de forma secundaria con respecto ao seu ligando nativo. A resposta de *E. faecalis* ás moléculas de sinalización de bacterias gram-negativas podería ser relevante en biofilmes polimicrobianos, onde *E. faecalis* estaría en contacto estreito con bacterias capaces de producir estas moléculas.

A investigación, mediante transcrición inversa e PCR cuantitativa, utilizando o método de cuantificación relativa (ΔΔCt), dunha selección de transcritos relacionados co QS mediado por péptidos, a formación de biofilmes e as respostas ao estrés da membrana, revelou varias regulacións asociadas a cada unha das cepas estudadas. Nas cepas ATCC 29212 e UmID7 observouse un aumento bioloxicamente relevante (máis do dobre) dos niveis dos transcritos relacionados con QS, o que suxire un aumento da virulencia destas cepas en presenza de AHLs. As regulacións observadas para a selección de xenes asociados á formación de biofilmes non se correlacionou co fenotipo observado ao avaliar a biomasa nesas mesmas cepas. Esta observación apunta á posibilidade de que a rede de xenes regulados tras da exposición ás AHLs sexa máis ampla que a abranguida mediante PCR cuantitativa. O uso de técnicas complementarias sería útil para conseguir unha mellor comprensión dos cambios que se producen nos biofilmes en presenza das AHLs. Os xenes de resposta ao estrés da membrana só se atoparon regulados de forma diferencial nunha das cepas de *E. faecalis*, UmID7, con aumentos relevantes nos seus niveis de expresión en presenza de AHLs, especialmente AHLs de cadea longa, o que podería levar á indución de fenotipos máis virulentos nesta cepa.

Posteriormente, seleccionouse a cepa *E. faecalis* ATCC 29212 para realizar máis estudos co fin de dilucidar os mecanismos subxacentes aos cambios no comportamento do biofilme observados en resposta ás AHLs. En primeiro lugar, os biofilmes de *E. faecalis* ATCC 29212 expuxéronse a AHLs de maneira individual, mostrando a AHL C_{18}-HSL como a única capaz de inducir un aumento significativo do biofilme por si mesma, segundo se avaliou mediante a tinguidura con CV. Tamén se observou un aumento da cobertura do biofilme despois da exposición a C_{18}-HSL usando microscopía confocal. O aumento do biofilme atribuíuse á presenza de C_{18}-HSL na súa forma intacta, xa que a exposición a unha C_{18}-HSL hidrolizada, co seu anel lactona aberto, daba lugar ao mesmo fenotipo que o biofilme non exposto a ningunha AHL. Este resultado suxire que a estrutura do anel lactona pode ser necesaria para inducir as respostas observadas nesta bacteria. Para afondar no coñecemento dos cambios experimentados durante o aumento do biofilme en *E. faecalis*, xeráronse biofilmes a partir dos que obter o proteoma completo de *E. faecalis* e realizar proteómica "shotgun", utilizando análises de espectrometría de masas para a identificación de proteínas. Ao examinar os perfís proteómicos da cepa ATCC 29212 en presenza de C_{18}-HSL, as proteínas máis abundantes non se alteraron, como se observa ao comparar o número de espectros de cada proteína identificada nas mostras control e nas mostras tratadas con C_{18}-HSL. Estes resultados suxiren que sería máis apropiado

escoller un método de comparación que permitise unha avaliación máis precisa das proteínas presentes en baixa abundancia nas mostras, xa que o número de espectros representa fielmente as proteínas presentes en alta abundancia. Non obstante, a aparición de dúas proteínas hipotéticas describiuse exclusivamente nas mostras expostas a C_{18}-HSL: A961_2010 e A961_692. A identificación destas proteínas na base de datos UniProt e no software DAVID deu como resultado unha anotación prevista para A961_692 como unha proteína reguladora da transcrición pertencente á familia YebC/PmpR de reguladores transcripcionais de unión ao ADN. Ata o de agora, non existe ningunha evidencia empírica sobre o papel destas proteínas no xénero *Enterococcus*. Non obstante, describiuse que unha proteína pertencente a esta familia regula procesos de QS en *Pseudomonas aeruginosa* (Liang et al., 2008). Estes resultados abren a porta a continuar co estudo das respostas deste supuesto regulador transcripcional á presenza de AHLs noutras cepas de *E. faecalis*, xa que previamente se observou que podían presentar circuítos reguladores moi diferentes. En conxunto, estes resultados apuntan a un papel ata agora non descrito das AHLs na regulación da formación de biofilmes de *E. faecalis*.

Os resultados obtidos nesta tese parecen indicar que o papel das AHLs ou moléculas similares ás AHLs foi subestimado no contexto da microbioloxía oral. Estas moléculas preséntanse como un obxectivo prometedor para a modulación da formación de biofilmes *in vitro*, e proponse a realización de estudos *in vivo* para validar esta tecnoloxía como unha alternativa factible aos antimicrobianos para o coidado da saúde oral humana. A confirmación do efecto inhibidor sobre os biofilmes da inhibición das AHLs en comunidades de biofilmes dominadas por taxons gram-positivos levou á investigación das interaccións entre as AHLs e a bacteria gram-positiva *E. faecalis*. A indución inequívoca da formación de biofilmes temperáns en varias cepas de *E. faecalis* en presenza de AHLs de cadea longa induciu máis investigacións destinadas a identificar os mecanismos moleculares subxacentes a estes cambios. En conxunto, os resultados aquí presentados contribúen á crecente evidencia de que as AHLs, ou incluso moléculas similares ás AHLs, xogan un papel importante no medio oral, e concretamente no biofilm oral.

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Annexes. Publications derived from the PhD thesis

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Publication 1.

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Ecological approaches for modulating oral biofilms as a novel strategy for the treatment or prevention of oral diseases

Ecological modulation of oral biofilms: novel approaches to treat and prevent oral diseases

Ana Parga and Ana Otero*

Department of Microbiology and Parasitology, Faculty of Biology, Campus Vida, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, SPAIN

(*) Author for correspondence. anamaria.otero@usc.es

Abstract

Oral diseases are now viewed as the consequence of a shift in the balanced relationship of the oral microbiota with the host, resulting in the increase of species and functions associated with disease. Traditional oral treatments aiming at resetting the dental plaque are often ineffective. This lack of success is partially due to the stability of the oral microbiota, which restores rapidly after treatment, and to the difficulties in effectively reaching and penetrating the dysbiotic dental plaque. Therefore, there is an increasing need for developing novel, more ecological, targeted approaches focusing on restoring the microbiome equilibrium and curving the self-activation loops that drive dysbiosis. Among the different ecological strategies that are being explored and have been reviewed here are pre and probiotics, phage therapy, antimicrobial peptides, and quorum sensing inhibition. Special attention should be paid to those that seem to be already involved in maintaining the homeostasis of the healthy oral microbiota. These strategies have yielded promising results *in vitro*, but *in vivo* trials are mandatory before the potential of these novel ecological strategies targeting to maintain and restore the equilibrium of the oral microbiota can be confirmed.

Keywords (5-10)

Oral biofilm, dysbiosis, probiotics, prebiotics, antimicrobial peptides, phage therapy, quorum sensing inhibition

In review

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1. Introduction

The advent of the -omics era in the field of oral microbiology, thanks to the increase in the potency and availability of high throughput sequencing techniques [1], has completely changed our concept of the microbial etiology of oral diseases. Oral diseases are now viewed as the consequence of a shift in the balanced relationship, or symbiosis, of the oral microbiota with the host, increasing species and functions associated with the diseases, a process generally known as dysbiosis [2, 3]. Therefore, caries and periodontitis, the two major oral diseases, result from a "regime shift" driven by carbohydrate consumption and acidification and plaque accumulation, respectively [3]. Additionally, the symbiosis with commensal plaque microbiota has beneficial effects on the host, acting as a barrier to opportunistic pathogens and helping maintain homeostasis in the oral cavity through metabolic and immunomodulatory activities [4, 5].

Traditional oral treatments are based on correcting dietary habits, improving oral hygiene, and fluor treatment for caries and mechanical dental plaque removal combined, in some cases, with antibacterial agents for periodontal diseases. These treatments, aiming at resetting the dental plaque, are often ineffective, partially due to the stability of the oral microbiota, which restores rapidly after treatment [6], and to the difficulties in reaching and penetrating the dysbiotic dental plaque. Although the use of antiseptics such as chlorhexidine gluconate or triclosan has some unarguable clinical benefits (reviewed by Serrano et al. [7]), these compounds may have undesirable side effects, including the down-regulation of oral keratinocytes and fibroblasts [8] or the reduction of the protective barrier against pathogens provided by the commensal microbiota [4]. For chlorhexidine, the most common oral antiseptic, it has been demonstrated that its indiscriminate antimicrobial action may cause major shifts in oral microbiota and metabolic activity, increasing the abundance of pathobionts, leading to more acidic conditions, or lowering nitrate availability [9, 10].

In this perspective, there is an increasing need for the development of novel, more targeted treatments, and preventive measures that should aim at maintaining a healthy, microbially diverse resident microbiome, promoting its resilience to the "disease drivers" and curving the self-activation loops that drive to dysbiosis [3, 8, 11–13]. Given the prevalent functional redundancy of microbial groups in the oral environment, the novel strategies should also target key metabolic activities and the expression of virulence factors rather than focus on specific groups of microbes. In this review, we will provide a general view of the novel strategies being explored for the targeted modulation of the oral microbiota. Some of these strategies seem to be already playing an important role in the oral environment, such as antimicrobial peptides (AMPs), bacteriophages, and quorum sensing inhibitors. Pre and probiotics also represent a more ecological approach targeting the re-establishment of the holobiont equilibrium.

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2. Novel Biological strategies

2.1. Prebiotics

Prebiotics are non-digestible substrates that are selectively utilized by commensal microorganisms conferring a health benefit to the host [14]. The use of prebiotics for improving oral health has been focused mainly on caries prevention and treatment [15, 16]. A caries-prone oral environment provides a low pH and high availability of lipidic metabolites and dietary carbohydrates. These characteristics favor the proliferation of bacteria that will ferment those carbohydrates yielding acidic products, like lactic acid, that result in the demineralization of the enamel [12, 17, 18]. Additionally, an elevated carbohydrate intake can lead to a positive feedback acidification loop driving the selection of acidophilic, cariogenic pathogens [3].

In the last decades, cumulative evidence points to **modulating the alkalinogenic potential of dental biofilms** as a promising strategy for caries control. In a healthy biofilm, the metabolism of amino acids, peptides, proteins, and urea leads to the net production of alkali. Alkali production helps to increase the pH after acid production resulting from the dietary intake of fermentable carbohydrates. Thus, as we have introduced above, when this equilibrium is tipped towards a predominance of acid production, caries can occur. One of the prebiotic approaches to modulate this shift is the administration of compounds that, when metabolized, raise the pH of their surrounding environment, counteracting the acidogenic effect of cariogenic bacteria.

Arginine is an amino acid found in saliva in free form and in salivary peptides and proteins [18, 19]. It has been identified as the main agent responsible for the increase in salivary pH, and it has been proposed as an agent to promote oral health [18]. Arginine is produced *de novo* from citrulline and from protein turnover, but it also enters the mouth with the dietary intake. The microbial degradation of arginine comprises several routes, the most common one being the arginine deiminase system (ADS) that yields citrulline, ornithine, CO_2, ATP, and ammonia [18] (**Figure 1**). This route is present in certain bacteria in supragingival biofilms, such as streptococci of the mitis group (*Streptococcus sanguinis, Streptococcus gordonii, Streptococcus parasanguinis, and Streptococcus mitis*), and some lactobacilli and *Actinomyces* strains [17, 20]. This production of ammonia results in a pH increase in the surroundings of these oral bacteria and protects them against acid. Additionally, the neutralization of the biofilm favors the persistence of ADS-positive bacteria and makes them competitive against aciduric and cariogenic microorganisms [21]. In fact, low levels of arginine deiminase activity in saliva and plaque have been proposed as indicators for caries in adult subjects [22]. Arginine has been administered with fluoride in dentifrices for over a decade with promising results and without negative side effects [16]. Arginine seems to have synergistic effects when combined with fluoride; this combination can suppress exopolysaccharide production, inhibiting biofilm formation [23], and it is generally an effective approach to restoring the oral microbial equilibrium (revised in Zaura and Twetman [12])., the arginine-fluoride combination has shown to inhibit *Streptococcus mutans* while

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favoring *S. sanguinis,* a species that is ADS positive and thus can use the available arginine to produce alkali [23]. At the same time, maintaining those oral niches colonized by streptococci exerts an ecological pressure against colonization by bacteria that thrive in alkalinogenic environments, such as the periodontopathogen *Porphyromonas gingivalis* [23]. However, the long-term impact of this strategy has yet to be confirmed. A feature to consider regarding the use of arginine as a prebiotic is that this amino acid is also a substrate of the nitric oxide synthase, i.e., a precursor of nitric oxide (NO) (**Figure 1**). NO can act as an antimicrobial agent but also be involved in the pathogenesis of periodontitis [24]. The incredibly delicate equilibrium between NO beneficial and detrimental effects on oral health should be further studied in the context of ecological modulations in this environment. With this in mind, prebiotic administration in caries patients at risk of developing other oral diseases or with other oral diseases already ongoing must be done cautiously, choosing the most beneficial strategy for each patient, and avoiding the risk of promoting further dysbiosis states.

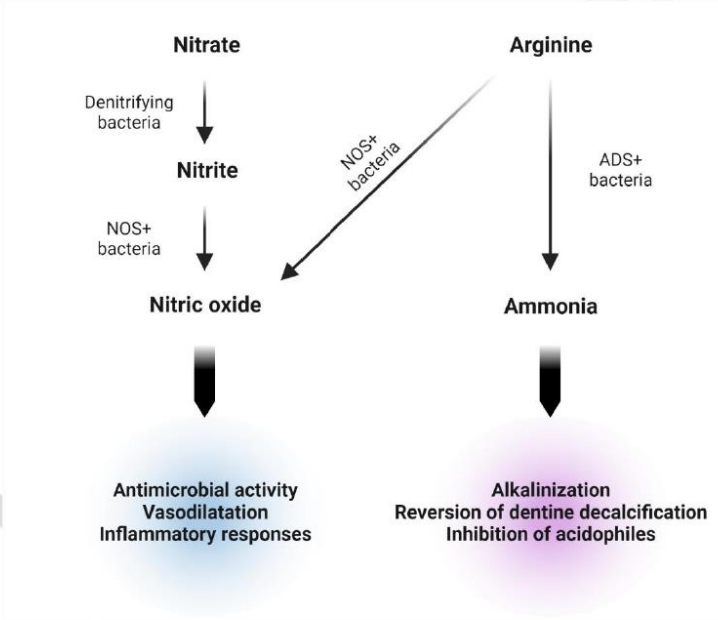


Figure 1. Convergence of different bacterial metabolic pathways in the production of nitric oxide. Nitrate reduction can lead to nitric oxide generation (left). Arginine plays a role in pH homeostasis via the arginine deiminase (ADS) pathway (right), and in nitric oxide synthesis, via the nitric oxide synthase (NOS) pathway.

Although its applicability as a prebiotic has been only recently suggested, evidence of the role of **urea** metabolism in acid neutralization in dental plaque goes way back [25]. Similar to the metabolism of arginine, the metabolism of urea mediated by the enzyme urease results in the production of ammonia

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and the subsequent increase in the pH of the biofilms [17]. This metabolic route is present in some oral bacteria such as streptococci from the salivarius group (*Streptococcus salivarius* and *Streptococcus vestibularis*), *Actinomyces naeslundii,* and *Haemophilus* spp. [17, 20]. Expression of urease is up-regulated at low pH as a response to pH stress in these bacteria [17]. Urea-containing mouthwashes were already used decades ago to reduce lactic acid production in the mouth and inhibit the development of new caries in individuals with active caries [26]. In addition, several studies have linked low levels of urease activity in dental plaque with caries [27–29]. Despite this evidence, the supplementation with urea as a prebiotic for anticaries purposes has not been widely studied since and needs further assessments to establish its efficacy and safety [30].

Nitrate is obtained via dietary intake, mainly from vegetables, and influences the oral microbiota of the host [17]. Nitrate is absorbed into the bloodstream, but an important portion is concentrated back in the saliva, where it will be reduced to nitrite by facultative anaerobic oral bacteria, with members of the genera *Neisseria, Veillonella, Actinomyces,* and *Rothia* showing the greatest nitrate reductase activities [17]. Nitrate reduction depends entirely on the commensal oral bacteria, as humans cannot perform this reaction [17]. Due to intraindividual microbiome variation, not all hosts will have the same net nitrate-reducing capacity [31]. In fact, nitrate-reducing bacteria are more common in individuals with lesser caries episodes [32]. Prebiotic supplementation with nitrate can help increase nitrate-reducing bacteria over time, thus increasing available nitrite and NO [31, 33] (**Figure 1**). Among its systemic beneficial effects, nitrite helps lowering blood pressure [17], and NO exerts antimicrobial activities against exogenous bacteria [17]. Nevertheless, as stated above, NO can also promote periodontitis progression [34]. Although more investigations are needed, studies show that nitrate supplementation prevents the pH drops resulting from carbohydrate metabolism [31], pointing to the potential efficacy of this prebiotic strategy. Prebiotic nitrate has been proven to change oral microbiota by reducing the abundance of genera related to caries, halitosis, and periodontal disease and inducing a more efficient nitrate reduction in the oral cavity [31]. The reduction of halitosis-related genera could be explained by the differential energetical costs of nitrate versus sulfate reduction. The latter is less efficient, and thus nitrate availability should limit sulfate metabolism, which yields volatile sulfur products related to halitosis [33]. It should be noted that nitrate and nitrite concentrations in saliva have been reported to be significantly higher in periodontitis patients [35], which can be the result of a host defense response, as it is known that salivary glands can respond to inflammation processes, such as periodontitis, by increasing salivary protective characteristics [36]. Thus, the nitrate and nitrite concentration increment in patients with periodontitis may correspond to the host defense response. One interesting aspect to consider when administering nitrate as a prebiotic would be its delivery through increased dietary nitrate-rich vegetables. The alkalinity of nitrate-rich foods and the presence of co-factors required for the reduction of nitrate could enhance the activity of nitrate-reducing bacteria, thus increasing the viability of this strategy.

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Other compounds studied because of their cariostatic potential are sugar substitutes such as **sugar alcohols**. Sugar alcohols, such as xylitol, are a type of low molecular weight polyols that cannot be metabolized by most bacteria in dental plaque [17, 37]. They are very popular as sugar substitutes in food products and beverages, as they confer moderated sweetness but do not translate into sugar spikes after consumption [37]. The latter characteristic, added to the reduction in available fermentable carbohydrates, has set the basis for considering sugar alcohols as prebiotics for caries control. The action mechanisms behind the cariostatic effects of sugar alcohols include the increase in buffering capacity and pH of the oral biofilm and the restoration of the enamel de-/remineralization equilibrium [37]. Specific action mechanisms on cariogenic bacteria, specifically mutans streptococci, including inhibiting insoluble glucan synthesis from sucrose and decreasing mutans streptococci cell counts in the mouth [12]. Nevertheless, even for the most studied sugar alcohols used as sugar substitutes, i.e., xylitol and erythritol, effects on caries control remain overall inconclusive [12, 38]. Moreover, it has been brought to attention that the cariostatic results obtained with chewing gums containing sugar alcohols cannot be wholly attributed to the effect of said compounds but at least partially to the salivary flow stimulation derived from the chewing itself, even when the gum does not contain sugar alcohols [39]. Moreover, the low compliance in patients limits the feasibility of sugar alcohols as prebiotic agents because of the long-term daily use required [40].

The strategy that has been proven more effective in the search for prebiotic agents to maintain oral health is the combined administration of prebiotics and probiotics so that the promotion of health-associated or beneficial bacteria is even higher [41]. Nevertheless, the application of personalized treatments for either solo or combined approaches has been recommended [15]. The complexity of the oral cavity is multiplied when studying different individuals, as each has its particular ecosystem. Moreover, regarding the previously commented approaches, the acid/alkali equilibrium in the oral cavity is highly dependent on the patient, as responses to changes in pH shifts have also been described to be variable between individuals [15]. Supplementation with exogenous molecules in the oral context will produce unique responses in each case. As the ulterior motive for prebiotic therapy is to ameliorate the health status of the host, choices on the specific treatment to recommend must be made on a personalized basis, together with pre-treatment evaluations of risk factors for developing oral diseases different from the ones being targeted with the administration of the prebiotic.

2.2. Probiotics

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [42]. Their application for oral health has been gaining attention for the past decades, and several strategies have been tested [43]. One of the principles underlying probiotic use is that the probiotic microorganisms should belong to the natural microbiota to establish themselves as residents in the targeted environment [44]. Thus, they would occupy the desired ecological niche

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so that other less beneficial species cannot colonize it or even displace these potentially harmful microorganisms when infection or disease is already ongoing [44]. The aim of a probiotic is to help achieve and maintain the ecological equilibrium. Although the complete picture of how probiotics help restore the ecological balance is not fully known, several **action mechanisms** have been described [44, 45] (**Table 1**).

The first direct effect of probiotic administration is the **competition with other bacteria**. This competition takes place both in terms of physical emplacement in a specific ecological niche and as a competition for the available resources found there [13, 16]. Once the probiotic is adhered to an oral surface or integrated into the oral biofilm, it will modulate its surrounding environment by modifying **the adhesion of other bacteria** to oral surfaces and in between them, i.e., modulating biofilm formation (revised in [16]). This modulation can be achieved through the intrinsic characteristics of the probiotic; this is the case when the cells possess surface components that prevent harmful bacteria from adhering to them. Another strategy for hampering pathogenic biofilm formation is the selection of probiotic bacteria that produce adhesion inhibitors [44, 45]. While several examples of probiotics inhibiting periodontopathogen growth or reducing cell counts have been described, most of the mechanisms behind these effects are still unknown (revised in [43]). A commonly hypothesized action mechanism of probiotics is the production of **antimicrobial molecules** (revised in [43]). There are some reports on the production of enzymes with anti-biofilm activity (i.e., lipase production [46]). Production of acid and H_2O_2 with activity against red-complex bacteria has also been described [47].

A crucial indirect action mechanism of probiotics is the **modulation of the immune responses** of the host. These responses have been mostly studied in the gut, where probiotics have been proved to alter the balance of pro-inflammatory and anti-inflammatory cytokines secreted by epithelial cells [45]. As oral probiotics gained attention, studies have focused on their effect on immune regulation in the oral environment. Effects on immune responses range from reducing inflammatory factor production by oral pathogens to the upregulation of the host's defense mechanisms (revised in [43]). Reductions in the production of pro-inflammatory factors such as tumor necrosis factor and interleukins (IL-1β, IL-6, IL-8, IL-17) by oral epithelial cells is one of the most recurrent responses described when assessing the effects of oral probiotics [43]. In some probiotic formulations, these decreases in pro-inflammatory factors have been linked to the production of arginine deiminase [48], an enzyme that can inhibit NO synthesis (**Figure 1**). Another interesting effect of probiotics is the reduction of pathogen-induced cytokine production and the antagonization of cytokine-induced cell proliferation and apoptosis (revised in [43]).

Regarding the effects on the hosts' immune system, probiotics have been described to increase the production of anti-inflammatory factors such as interleukin 10 (IL-10) [49, 50]. The expression of β-defensins, the antimicrobial peptides that act at the epithelial level, is also increased by the action of some probiotic strains [51]. Additionally, increases in hemocyte density *in vivo* have been attributed

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to the use of lactobacilli-based probiotics [52]. Attenuation of immune responses via the inhibition of nuclear factor kappa B (NF-κB) and the activation and inhibition of NO production in macrophages has also been described in response to probiotic uptake [53].

Considering these action mechanisms just described, certain **characteristics are interesting to find when looking for potential probiotic species**. The most immediate characteristic related to the sustainability of the potential probiotic is the ability to adhere to oral surfaces successfully or to become integrated into preformed oral biofilms, competing for binding sites or co-aggregating with other microorganisms to remain viable in the mouth [44]. *A priori,* ideal probiotic candidates would be bacteria naturally occurring in the oral cavity, as they would be expected to better adapt and resist the oral environmental conditions, adhere to soft or hard oral tissues/surfaces, and be integrated with the normal microbiota residing there [44]. Moreover, they should not entail a bacterial challenge for the immune system of the oral environment. Another desirable characteristic of a probiotic is the production of antimicrobial substances against harmful bacteria [44]. Once they have been selected based on their biological characteristics, probiotics must preserve their viability and functional properties after enduring the manufacturing processes necessary to administrate them. These processes include undergoing encapsulation, extrusion, lyophilization, and even processing of food products they may be integrated into.

Considering these characteristics, we will go over **the most common genera used as probiotics in the oral field**. Currently, it is found that probiotics with the potential to contribute to oral health are concentrated in the genera *Lactobacillus, Bifidobacterium, Weissella,* and *Streptococcus,* as well as certain scattered species like *Bacillus subtilis* and the yeast *Saccharomyces cerevisiae* [43]. The most common oral probiotics are members of the genus ***Lactobacillus*** [13, 44]. They are classical gastrointestinal probiotics and common inhabitants of the mouth [12, 17], hence their translation to the oral field to assess their potential as oral probiotics. In addition, their antimicrobial activity has been extensively studied, especially in gastrointestinal probiotic applications [44], and against oral yeasts [16, 44]. Numerous trials have examined the effects of lactobacilli administration for oral health improvement, generally describing promising results, as inflammation and pathogen load are generally reduced in the presence of lactobacilli-derived probiotics (**Tables 1, 2**). Several studies have tested the ability of probiotic lactobacilli to prevent caries [16] with successful results in the reduction of *S. mutans* counts, at least for the duration of the treatment time. Lactobacilli have also been tested for other oral diseases; a study on animal models with induced apical periodontitis has described a reduction in tissue inflammation following a lactobacilli-based probiotic [54]. These results open the door to further studies on the clinical practice of using probiotics as adjuvants to treat periodontitis.

Bifidobacterium is another genus traditionally used as a gastrointestinal probiotic [12]. Its application in the oral field followed that of lactobacilli, as *Bifidobacterium* can also be found in the oral cavity [17]. Administration of *Bifidobacterium*-containing probiotics lowered bacterial counts of several oral

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pathogens and regulated immune responses in *in vivo* studies and clinical trials (revised in Zhang et al. [43]). Some bifidobacteria-based probiotics have undergone clinical trials that demonstrated their ability to lower clinical signs of gingivitis [55]. Despite these promising results, it is worth noting that species of both the genus *Lactobacillus* and *Bifidobacterium* can be highly acidogenic and thus have the potential to initiate dental caries or aid in the progression of carious lesions [12, 44]. This concern has been brought up when examining the efficacy of probiotics containing these genera and has prompted the search for alternatives in other groups.

Strains belonging to the genus ***Weissella*** have also been examined as oral probiotics. Members of this genus have been isolated from human saliva [56], although they are mainly found in fermented foods [57]. They comprise both potentially pathogenic and probiotic species [43]. Probiotic strains of the species *Weissella cibaria* have been described to have antibiotic activities, as well as the ability to regulate immune responses (revised in Zhang et al. [43]). Probiotic *W. cibaria* has a great co-aggregating capacity with oral pathogens [43], which is thought not to interfere with pathogenic colonization but suppress the growth of harmful bacteria.

Streptococci are part of the normal microbiota of the human mouth. They are primary colonizers, providing the necessary elements for a healthy ecological progression in the oral biofilm and the attachment of subsequent species [17]. However, some species of *Streptococcus* are potentially pathogenic to humans and have been linked to oral disease [17]. This is the case of *S. mutans* and caries development. In fact, plenty of probiotics are evaluated against *S. mutans* to assess their potential to treat and prevent caries [16]. Nevertheless, among the genus *Streptococcus* we can also find strains with probiotic potential. This is the case, for example, of *Streptococcus dentisani*, a strain isolated from the oral cavity of caries-free individuals that has shown a great caries-protective capacity, thanks partially to its acid-buffering abilities [58]. Probiotic streptococci can modulate periodontal pocket recolonization, as their administration has been described to reduce and maintain low levels of anaerobic species and black-pigmented bacteria [59]. This evidence points towards a possible application for periodontal disease management.

Finally, the emergence of studies on **recombinant probiotics** also offers interesting results in increasing efficacy against oral pathogens or minimizing problems associated with certain probiotics. This strategy is based on the genetic modification of existing probiotic strains so that they express pathogen-related proteins to stimulate pathogen recognition by the immune system [60, 61]. So far, a few in vivo studies point to the success of these modified probiotics [60, 61]. However, their applicability in humans is uncertain, as genetically modified organisms are not always easily accepted as medical treatments.

The application of probiotics in the oral field for the prevention and treatment of dental caries and periodontal disease has been examined by individual studies and meta-analyses. There seems to be a tendency towards higher success rates in their application for periodontal disease control [62], [9]

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although meta-analyses find that probiotics do not always significantly improve oral health parameters related to the progression of these diseases [63]. Several factors contribute to discrepancies found among studies, such as the specific probiotic strains or combination of strains used, the probiotic administration modes and schedules, and the dosage [43]. The general lack of information on the most effective doses and application modes for oral probiotics contributes to the variability of outcomes observed [63]. Intraindividual differences are a big cause of variation too. As oral disease prognosis depends on the specific microbiota and immune system of each patient, probiotic therapy outcomes will also vary when administered to different patients [15]. Personalized treatments are already on the landscape of traditional oral treatments, and probiotic therapy should follow this trend [30]. Preceding studies on intestinal probiotics have brought up the need for tailored probiotic treatments to achieve better colonization rates and increase general effectiveness [44]. The objective of obtaining efficient, personalized probiotic treatments will probably arrive with the expansion of combinational therapies. Using different strains and even species combined in the same probiotic treatment could lead to synergistic effects, as dental plaque is an extraordinarily complex niche with multiple species inhabiting it. Genetic modification of probiotics could also be a successful approach to treating oral diseases [60, 61] once the safety of these probiotics is proven for their use in humans.

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Table 1. Effects of probiotic bacteria on oral pathogens.

Effect	Probiotic bacteria	Type of study			Proposed action mechanism	References
		In vitro	*In vivo*	Clin. trials		
Reduction of oral pathogen presence in oral tissues and exudates	Lactobacilli			✓	-	[64–66]
	Bifidobacteria			✓	-	[65]
	W. cibaria		✓	✓	-	*In vivo* [67]; clinical trials [68]
Change of the ratio between aerobic and anaerobic bacteria	Lactobacilli		✓		-	[69]
	Bifidobacteria		✓		-	[70]
Reduction of colonization capacity of oral pathogens	Lactobacilli		✓		-	[51]
	S. cristatus	✓			-	[71]
Inhibition of oral pathogen growth	Lactobacilli	✓			Release of postbiotics (lipase) [46]	[46, 52, 66, 72, 73]
	Bifidobacteria	✓			-	[74]
	S. dentisani	✓			-	[58]
	W. cibaria	✓			Production of acid, H_2O_2, and N-acetylmuramidase	[47]
Reduction of biofilm formation in oral pathogens	Lactobacilli	✓			Release of postbiotics [75] and production of lipase [46]	[46, 66, 75]
	Bifidobacteria	✓			-	[76]
	S. cristatus	✓			Production of arginine deiminase	[48]
Reduction of oral pathogens' adhesion to cells	Lactobacilli	✓			-	[73]
	Bifidobacteria	✓			-	[74]
	S. dentisani	✓			-	[58]
	W. cibaria	✓			-	[56]
Down-regulation of virulence- and adhesion-related factors in oral pathogens	Lactobacilli	✓			-	[73, 75, 75, 77]
	S. cristatus	✓			Production of arginine deiminase regulates fimbrial gene expression of *P. gingivalis*	[48]
Change of cell wall structure of *P. gingivalis*	*S. dentisani*	✓			-	[58]
Induction of cell lysis in *F. nucleatum*	*S. dentisani*	✓			-	[58]

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Table 2. Effects of probiotic bacteria on immune response regulations and disease progression

Effect	Probiotic bacteria	Type of study			Proposed action mechanism	References
		In vitro	*In vivo*	Clin. trials		
Inhibit/reduce the expression of pro-inflammatory factors (interleukins, cytokines, interferon-g)	Lactobacilli	✓	✓	✓	Production of arginine deiminase [69]	*In vitro* [51, 56, 72]; *in vivo* [69]; clinical trials [78]
	Bifidobacteria		✓	✓	-	*In vivo* [50, 70]; clinical trials [55, 74]
	Streptococci	✓			-	[49, 79]
	W. cibaria	✓	✓		-	*In vitro* [56]; *in vivo* [67]
Increase the expression of anti-inflammatory factors	Bifidobacteria		✓		-	[50]
	S. dentisani	✓			-	[49]
Raise the expression of β -defensin, TLR4, and CD4 in the gingiva	Bifidobacteria			✓	-	[74]
Reduction of bone resorption and inflammation in apical periodontitis	Lactobacilli		✓		-	[54]
Increase in hemocyte density upon *P. gingivalis* infection	Lactobacilli		✓		-	[52]
Decrease the expression of anti-inflammatory factors (IL-10)	*W. cibaria*		✓		-	[67]
Reduce the number of $TRAP^+$ cells and infiltrating inflammatory cells	Lactobacilli		✓		-	[80]
Raise the level of mouse β-defensin mRNA in gingiva, tongue, and saliva	Lactobacilli		✓		-	[51]
Antagonizes proliferation and apoptosis induced by *P. gingivalis*	Lactobacilli	✓			-	[81]
Inhibit NF-kB activation and NO production in macrophages	*W. cibaria*	✓			-	[53]

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2.3. Phage Therapy

Bacteriophages or phages are viruses with the capacity to infect and kill bacterial cells. Due to their specificity and self-replicating nature, the use of bacteriophages for the treatment of certain multi-drug resistant bacterial infections, a strategy also known as phage therapy, is gaining increasing attention [82, 83], and its efficacy has been already tested in the clinical practice [84]. The existence of phages in the oral environment has been known for decades, and they have been found in oral samples, including phages of important oral pathogens, such as *Fusobacterium nucleatum* and *S. mutans* [8, 85–90]. As in many other microbial communities, oral phages are thought to play a significant role in shaping the oral microbiome by influencing the species equilibrium and acting as mediators of genetic exchange [87], but the ecological involvement of bacteriophages in oral diseases has not been completely clarified yet. As HTS techniques have been implemented for oral studies, a broader picture has been constructed, and putative phages of numerous genera belonging to the major bacterial phyla of the oral cavity have been described [91]. In fact, a significantly higher abundance of lytic phages was found in the subgingival plaque of individuals with periodontal disease compared to healthy subjects, suggesting that phages play a critical role in modulating the microbial community and contributing to periodontal disease progress [92]. Transcriptome analysis of the oral bacteriophage community performed on healthy donors and periodontal disease patients hinted at a more active role of lytic phage molecules in the latter [93]. However, more studies are needed to establish the relevance of viral gene expression on oral disease onset and progression.

Bacteriophages can have two life cycles: a lytic cycle in which phage infection leads to the death of the bacterial host, and a lysogenic cycle in which viral DNA is integrated into the bacterial chromosome, replicating "silently" within the host without killing it. Lytic bacteriophages are the most suitable for developing phage-based treatments, as lysogens (bacteria containing virus with a lysogenic cycle) present increased pathogenicity and elevated resistance to phage therapy [8]. Interest in the use of phage therapy resides in the bactericidal activity of the bacteriophages but also in the possibility of using them as "Trojan horses" to deliver genes encoding for enzymes or toxins to the bacteria [12]. The ultimate purpose is for these genes to be translated and their products produced by the host bacteria, ultimately inflicting damage on it. These enzyme- or toxin-encoding genes can either be naturally present in the genome of the bacteriophage or be genetically engineered. Additionally, isolated phage enzymes, such as the endolysins used to digest the bacterial walls and spread to neighbor cells, have been explored as an antimicrobial strategy [82] and have also been proposed as a viable strategy *in vitro* against oral pathogens [90].

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As stated previously, it is common to find bacteriophages in the oral microbiome [88, 89, 91], including those with activity against bacterial genera implicated in oral health and disease. Several studies have been conducted on bacteriophages' use to restore the oral microbial equilibrium. Despite this, nowadays, investigations in oral phage therapy are mostly limited to *in vitro* studies, with few exceptions. *For example, Enterococcus faecalis* is one of the species in which more bacteriophages have been described, and a wide range of phages are available for the control of this species in the oral context (revised in Szafrański et al. [88]). The implication of *E. faecalis* in infections after root canal treatment and the incidence of multiresistant strains in this species has promoted the studies on alternative therapies against this bacterium. Different bacteriophages have successfully inhibited *E. faecalis* growth *ex vivo* in root canals [94, 95]. It is also interesting to assess the effect of phage therapy in biofilms, as these structures are linked to oral disease progression. There are a few reports on inhibiting *E. faecalis* biofilm formation using bacteriophages in *in vitro* experiments [95, 96] and *ex vivo* in root canals [95]. To the best of our knowledge, the only *in vivo* study on phage therapy against oral pathogens is the work of Xiang et al. [97] on the effect of a bacteriophage against an *E. faecalis* strain isolated from a periapical periodontitis lesion. Confirming the results of *in vitro* and *ex vivo* assays, an abdominal infection model and a periapical periodontitis model, both performed in rats, showed that treatment with the bacteriophage successfully decreased mortality and completely prevented periapical periodontitis, respectively [97]. Phage therapy was even more effective than antibiotics tested in the same conditions [97]. These results open the door to further *in vivo* studies to assess the feasibility of using phage therapy after root canal treatment as a prevention strategy and on ongoing infections as a treatment approach.

Mutans and mitis streptococci are common targets for bacteriophages, with a high number of phages active against *S. mutans, S. mitis, Streptococcus sobrinus,* and *Streptococcus oralis* (revised in Szafrański et al. [88]). Studies report higher lytic activity in bacteriophages targeting mutans streptococci than mitis streptococci [88]. Independent studies have found different *S. mutans* bacteriophages that can almost completely inhibit biofilm formation *in vitro* in *S. mutans* [98, 99]. Interestingly, several modified lytic enzymes obtained from streptococcal phages have been patented for oral health maintenance [100–102]. Additional *in vitro* studies on enzymes against streptococci described a lysin that was selectively active against 3 different streptococci; *Streptococcus pneumoniae, S. oralis,* and *S. mitis* [103]. Phage lysins appear as a promising new antimicrobial strategy that can also synergically act with antibiotics by increasing cell wall permeability and decreasing the antibiotic minimum active concentration [82]. Further *in vivo* and subsequent safety and clinical trials await before this strategy can be further implemented.

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The evidence on phage therapy against other oral bacteria is mostly limited to *in vitro* studies with moderately successful results. Briefly, additional bacterial genera with reported bacteriophages comprise *Aggregatibacter, Fusobacterium, Lactobacillus, Prevotella, Treponema,* and *Veillonella* (revised in Szafrański et al. [88] and Steier et al. [90]). Among these, a limited number of species or even strains have been described to have specific bacteriophages [88, 90].

Phage therapy is a promising alternative or adjuvant to antibiotics [104], especially for root canal post-intervention [8]. The advantages of phage therapy include a low impact on the commensal microbiota, easy isolation and purification processes, the possibility to genetically engineer phages, and their increased efficacy on biofilms compared to conventional antimicrobial agents, as reported *in vivo* against *E. faecalis* [97]. Nevertheless, there are also some limitations associated with their use. First, the limited capacity of phages to effectively penetrate biofilms is a particularly relevant issue in the oral cavity. Their high strain-specificity calls for precise diagnostic procedures or, when these are not available, for the use of phage cocktails. Their potential immunogenicity and the risk of lysogeny have also been brought up as a general concern [83, 88, 105]. All these aspects call for further investigations regarding the safety and applicability of this strategy. To date, the lack of clinical trials limits the validation of phage therapy as a strategy to treat and prevent oral disease.

2.4. Antimicrobial Peptides

Antimicrobial peptides (AMPs) constitute a natural defense mechanism against pathogenic microorganisms. The production of AMPs has been described in pluricellular organisms, where they can also be called **host defense peptides**, and bacteria. AMPs produced by the latter are commonly addressed as **bacteriocins**. Bacteriocins comprise several subclasses, from which the lantibiotics, produced by lactic acid bacteria, have been the most extensively studied [82]. It has been demonstrated that human-produced AMPs specifically target microbes and contribute to the ecological equilibrium in the gut [13]. Bacteriocins have an additional role in providing ecological advantages derived from the elimination of competitor bacteria and the subsequent increase in available nutrients in their surroundings [82].

AMPs are short, often post-translationally modified polypeptides consisting of 12–50 amino acids synthesized via the ribosomal way. These peptides have varied structures that condition their mode of action, and they are ubiquitous in nature. The most common bactericidal mechanism of AMPs is the formation of pores in microbial membranes, although the targeting of intracellular components has also been proposed as responsible for the killing effects of AMPs [82, 106]. The affinity of AMPs for microbial membranes is due to their physicochemical

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characteristics: the AMPs are predominantly positively charged due to the presence of amino acids such as cysteine and lysine, and bacterial membranes tend to be negatively charged due to the presence of hydroxylated phospholipids (Gram-negative bacteria) and teichoic acids (Gram-positive bacteria) [107]. Contrarily, mammalian cells tend to be more neutral in terms of charge, and thus AMPs are much less likely to attach to them [106]. This selectivity makes them interesting as targeted therapeutics against pathogens, as they do not present high toxicity towards host cells (revised in Rios et al. [82] and Mai et al. [106]). After the initial attraction towards the microbial membranes, AMPs can be integrated into them or go through them to reach the bacterial cytoplasm [106]. AMPs tend to have an amphipathic structure that allows them to interact with microbial components and other innate defense molecules [17, 82]. AMPs are generally constituted by a C-terminal domain, responsible for pore formation in bacterial membranes, connected to an N-terminal domain, responsible for the binding to bacterial surface receptors. The integration of AMPs in the microbial membranes can disrupt the membrane structure, eventually causing cellular death. On the other hand, the permeabilization of the membrane allows for AMP leakage into the cytoplasm, where it can bind to nucleic acids and cellular components to disrupt the normal functioning of the microbial metabolism [106, 108].

The production of human AMPs has been described in the oral cavity in salivary glands, oral mucosa, and dental pulp. Some AMPs have also been found in gingival crevicular fluid (GCF) [17, 109]. There are three main types of AMPs described in the oral context. **Defensins** can be expressed constitutively in salivary glands or upon a bacterial-induced inflammatory signal [17] and exert antimicrobial activity against bacteria, fungi, and viruses [17, 109]. Human β-defensins (hBDs) can be released by the host's immune cells and have been detected in numerous oral mucosal surfaces and epithelia [17]. Another type of AMPs found in human salivary glands are **histatins.** Histatins are cationic, histidine-rich peptides with antibacterial and antifungal activities. They are active against proteases and adhesins of host and microbial origin and can also inhibit bacterial-induced cytokine production by binding to pathogenic outer membrane proteins [17]. Lastly, the LL-37 peptide is the only member of the **cathelicidins** that has been described in the oral environment in humans. LL-37 is produced in both epithelial cells and neutrophils and has antibacterial properties [17, 106]. In addition, the LL-37 peptide has immunomodulatory activities, as it can bind to inflammatory molecules of microbial origin and attract immune cells to the infection site [17].

Bacteriocin production by human oral bacteria is rather common, with members of the genera *Streptococcus, Lactobacillus, Fusobacterium,* and *Prevotella* presenting this activity [110]. Thus, many of these groups are considered to have probiotic potential, as we have revised in section

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2.2 of this chapter. Most of the studies on isolated bacteriocins for therapeutic approaches have been performed with the subclass lantibiotics [111].

AMPs have been presented as alternatives to antibiotics to treat microbial infections in humans for their selectivity towards microbial cells and less expected tendency to induce resistance. The AMPs accepted/approved by the FDA and already on the market are polymyxins B and E (colistin) and daptomycin [82]. Other natural AMPs have been tested as therapeutics *in vitro*, and some are currently undergoing *in vivo* assays and clinical trials [82, 112]. These efforts have focused on different infections, such as respiratory infections or medical devices-related infections. These investigations have brought to light some disadvantages of the use of natural AMPs, such as certain unspecific activity towards mammalian cells, which has been reported especially with defensins [113]. Some characteristics of natural AMPs may reduce their efficacy when used in the oral context, such as their susceptibility to protease action and subsequent loss of antimicrobial activity [82]. Fortunately, advances in the modification of peptide sequences have made possible the creation of synthetic or "targeted" antimicrobial peptides. These synthetic peptides possess advantageous characteristics regarding their stability, bioavailability, selectivity, and half-life [112, 114, 115].

Both natural and synthetic AMPs have been investigated for the treatment of oral diseases, both for their direct effects on microbes and their immunomodulatory properties. Several formulations containing AMPs are already commercialized for topical and mucosal infections outside the oral context [82]. However, in the oral field, most studies have been performed *in vitro* and have not yet reached clinical trials.

Natural AMPs have been linked to oral health, as they have been found in significantly higher proportions in the metagenomes of caries-free individuals [116]. Moreover, the distribution of strains with low susceptibility to AMPs has been correlated with **caries** progression [117]. Studies on natural AMPs point to a protective effect on oral health, acting against cariogenic pathogens [109]. On the other hand, synthetic AMPs against cariogenic species are mostly studied *in vitro* and mainly on planktonic cells, although reports of antibiofilm activities can also be found (revised in Niu et al. [109]). Two derivates of human β-defensin-3 (hBD-3) have proved their inhibitory activity on *in vitro* streptococcal biofilm formation [118, 119]. KSL-W, a synthetic peptide, has shown great stability *in vitro* in saliva, conserving its antimicrobial activity in this environment [120]. When developing a formulation that included KSL-W and cetylpyridinium chloride (CPC) for caries control, these two components displayed synergistic effects disrupting *in vitro* oral biofilms formed on hydroxyapatite (HA) discs [121]. Recently, the oral bioavailability of KSL-W has been improved by fusing this AMP with a peptide that binds to hydroxyapatite [122]. Another example of an AMP targeting a cariogenic pathogen is the synthetic peptide

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C16G2. C16G2 was engineered to improve its stability and antimicrobial abilities, and it has been proven to specifically target *S. mutans* in *in vitro* saliva-derived biofilms without affecting closely related non-cariogenic streptococci [115, 123]. Different formulations of this synthetic AMP have been tested in clinical trials for oral health purposes, with C16G2-based varnishes, mouth rinses, oral gels, and strips reaching phase II of clinical trials (identifiers: NCT03196219, NCT02044081, NCT02254993, NCT02509845, NCT02594254, NCT03004365 and NCT03052842, retrieved from the ClinicalTrials.gov database of the U.S. National Library of Medicine [124]). To the best of our knowledge, no further results have been made available on the progression of said trials.

Bacteriocins have also been tested as alternatives to antibiotics on **cariogenic species**. The lantibiotic nisin, for example, has been successfully tested *in vitro* against mono-specific biofilms of *S. mutans* [111]. However, when nisin was examined against a 3-species biofilm containing *S. oralis, S. gordonii,* and *A. naeslundii*, no antibiofilm activity was observed, and antimicrobial action was moderate [125]. Interesting results have been obtained by Arslan et al. using the iron-free form of lactoferrin on dual-species biofilms formed onto saliva-coated surfaces. Lactoferrin was able to significantly reduce the biofilm of *S. gordonii-F. nucleatum,* and of *S. gordonii-P. gingivalis* [126]. Also, the peptide 1018 almost completely eliminated the biofilm formation of supragingival plaque samples cultured *in vitro* [127]. This evidence points towards a promising application of AMPs to control cariogenic biofilms.

Some AMPs may also potentially prevent **periodontal disease**-related microorganisms from attaching and further developing into a biofilm. Nisin, which action was already commented against oral streptococci, was shown to inhibit the growth of both early and late colonizers in saliva-derived biofilms formed *in vitro* [128]. Among the late colonizers assessed, *P. gingivalis, Prevotella intermedia, Treponema denticola,* and *Aggregatibacter actinomycetemcomitans* were the most sensitive to nisin. In addition, multispecies biofilms were reduced in terms of biomass and thickness when cultured in the presence of nisin [128]. Interestingly, the nisin effect on oral epithelial cells was assessed *in vitro*, showing no toxicity when used at concentrations showing antimicrobial and antibiofilm activities [128]. A hallmark of periodontal diseases is the inflammation that is common to all of them. The potential of AMPs as a strategy against these dysbiotic states resides partly in their immunomodulatory activities. Lactoferrin, already proposed for caries prevention, has also shown very interesting anti-inflammatory effects, attributed to its ability to sequester bacterial LPS [129]. Lactoferrin is, in fact, a component of several products destinated for oral health maintenance, with beneficial effects being demonstrated in several clinical trials [130, 131].

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Regarding oral **candidiasis**, AMPs have also been proposed as therapeutics, as many natural AMPs have antifungal activity. For example, histatin 5, naturally present in human saliva, has demonstrated antifungal activity against *Candida*. In particular, Rothstein et al. established that it was just a 12-amino acid fragment of this histatin that contained the antifungal activity, as they proved *in vitro* against several *Candida* spp. clinical isolates [132]. The *in vitro* inhibitory effect on *Candida* biofilms formed onto the materials from which dental devices are made was also demonstrated, with efficient results upon prolonged exposure [133]. Delivery of histatin 5 in gels for topical oral application has been tested *in vivo* in animal models, displaying protective effects against *Candida* infection and health restorative effects against pre-existing lesions [134]. These findings support the feasibility of histatin 5 as a novel therapeutic agent. More *in vivo* assays and clinical trials are needed to further validate these approaches for oral health maintenance and their application in patients at risk of oral candidiasis.

The application of AMPs to oral health relies on previous knowledge obtained from the treatment of other infections. This fact paves the way in multiple aspects of the investigations related to them, for example, their potential to be engineered to obtain improved features or their interactions with other therapeutic compounds, such as antibiotics. This combinational antibiotherapy has been successfully applied outside the oral field [82]. It is expected for this approach to be also tested for oral infections and diseases. As we have shown throughout this section, currently, the main limitation is the scarce evidence of the effectiveness of AMPs *in vivo* and in clinical trials, which will probably be overcome in the upcoming years, as several compounds are entering the pipeline for these procedures.

2.5. Quorum Sensing Inhibition

Many relevant pathogenic bacteria regulate their biofilm formation through cell density-dependent communication processes known as quorum sensing (QS) (**Figure 2**). Thus, QS processes have been proposed as potential targets for developing strategies to inhibit oral biofilm formation [135–137]. Furthermore, because strategies targeting QS do not interfere with bacterial growth, these approaches have been proposed to be less likely to induce bacterial resistance [135, 138–143].

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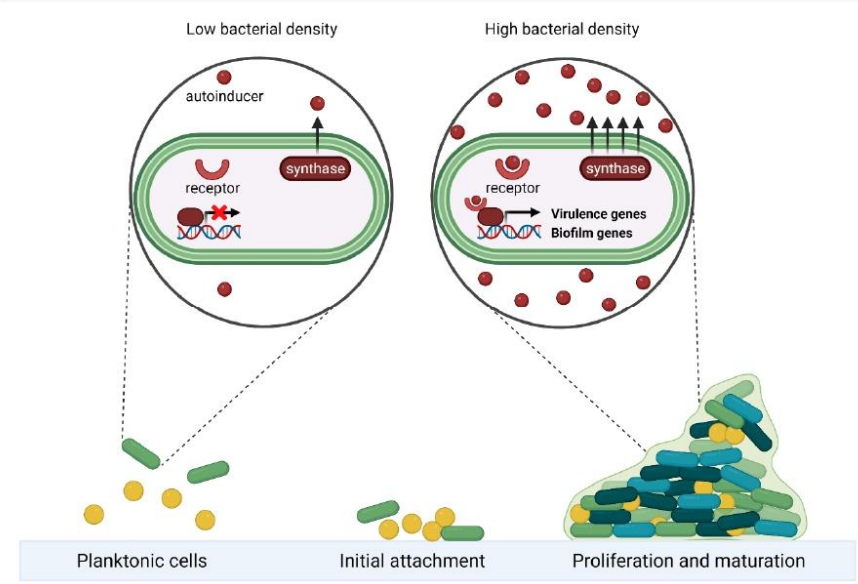


Figure 2. Quorum-sensing dependent regulation of gene expression of biofilm-related and virulence-related genes. Modified from Muras et al. [154].

Bacteria present different QS signals and systems. Furthermore, some bacteria that do not produce their own QS signals have receptors for QS molecules present in their surroundings or produced by other bacteria [144]. The relevance in the oral context of two of the most common QS signals: the autoinducer-2 (AI-2), produced by both Gram-positive and Gram-negative pathogens, and autoinducer peptides (AIPs), produced by Gram-positives, is traditionally accepted [145–148]. However, acylhomoserine-lactones (AHLs), the QS signals that are best characterized, have been excluded from the current paradigm of QS in dental plaque [146], even though multiple pieces of evidence suggest an important role of these signals in the oral cavity [145, 146, 149–151]. Prevention of bacterial coaggregation and reducing dental plaque are supposed to have beneficial effects on oral health. Thus, understanding the QS processes that are active inside oral biofilms is crucial to developing new strategies for preventing oral diseases. Among the three best studied QS signals, the **autoinducer-2 (AI-2)** is a group of signaling molecules present in both Gram-negative and Gram-positive bacteria and synthesized by the LuxS enzyme. LuxS, which is also involved in the detoxification of SAM and therefore has an important role in metabolism, is widely distributed [152, 153]. AI-2-mediated QS has been

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pinpointed as critical for biofilm formation [146–148, 154], and synthesis of AI-2 has been described in oral commensal and pathogenic bacteria (revised in Muras et al. [155]). AI-2 has been proposed as a starting point for the establishment of dysbiosis in the oral environment [146–148, 154], as its production is higher in pathogenic species than in commensal microbiota [146].

The **autoinducer peptides (AIPs)** are post-translationally modified peptides that Gram-positive bacteria can use as QS molecules. Its production has been described in oral streptococci, with the best-studied AIP being the competence-stimulating peptide (CSP) of *S. mutans.* The AIP of streptococci consists of a two-component transduction system: the specific signal binds to a membrane-bound histidine kinase sensor inducing the phosphorylation of response regulator proteins, ultimately regulating gene expression. Nevertheless, the role of CSP in biofilm formation remains inconclusive (revised in Muras et al. [155]).

Acylhomoserine lactones (AHLs), formed by a homoserine lactone ring (HSL) linked by an amide bond to a fatty acid of between 4 and 20 carbons, are the best studied and characterized QS signals [156]. AHLs are typically considered to be of Gram-negative origin, but their production by Gram-positive bacteria has also been described [157, 158].

AHLs have been traditionally excluded from the paradigm of cell-cell communication in the oral cavity due to the lack of success in detecting the production of AHLs by the main oral pathogenic species in pure cultures [145–148]. Recently, AHL production has been described by bacteria isolated from caries lesions [159, 160] and human tongue surface [161–163]. Additionally, the production of an AHL has been described in pure cultures of *P. gingivalis* [136]. *P. gingivalis* has also been shown to respond to AHLs by changing its protein expression profile and slowing down its growth [136]. Moreover, adding AHLs to an *in vitro* multispecies biofilm modified protease activity and lactic acid production and specifically increased the proportion of the orange-complex bacteria *Peptostreptococcus* and *Prevotella* [164]. In the light of this evidence, the role of AHLs in QS communication in the oral environment should be revised [136, 137, 155]

Given the importance of QS in biofilm development, these processes have been proposed as a target for the control of biofilm formation. The interference with QS, also known as quorum sensing inhibition (QSI), is a group of strategies that comprise the blockade of the QS signal receptor and direct effects on QS signal concentration, e.g., via enzymatic degradation, a strategy known as quorum quenching (QQ).

Inhibition of the AI-2 and CSP systems has been proved a successful strategy for inhibiting *in vitro* oral biofilms [135, 136, 165–170]. D-galactose reduced biofilm formation in *F. nucleatum, P. gingivalis, and Tannerella forsythia* by interfering with AI-2 sensing [170]. Similarly, D-ribose and furanone, both QS inhibitors, reduced bacterial load and bone destruction in a mice

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infection model of *P. gingivalis* [169]. The combination of ribose with xylitol was effective against *Streptococcus* biofilm formation [168], evidencing the importance of a global comprehension of biofilm ecology when looking out for biofilm inhibition strategies. When using a cell extract that interferes with AI-2 QS, Muras et al. [135] observed a significant reduction in *S. mutans* biofilm formation. Wang et al. [165] reported that several oral streptococci, as well as oral pathogens such as *P. gingivalis,* could interfere with the CSP system in *S. mutans,* reducing biofilm formation and bacteriocin production [165]. So far, these promising studies have been limited to *in vitro* investigations. Thus, further *in vivo* studies are needed before this approach can be a candidate for implementation in clinical practice.

Last, the inhibition of AHLs to control biofilm formation is of special interest since several inhibitors, and enzymes targeting these molecules have been reported [139–141]. An *in vitro* 6-species biofilm model containing *S. oralis, Veillonella parvula, A. naeslundii, F. nucleatum, A. actinomycetemcomitans,* and *P. gingivalis* responded to the addition of a QQ enzyme, Aii20J, by reducing the biofilm coverage and viability of the cells [136]. Furthermore, a recent *in vitro* study with 33 subjects conducted in our group reported that the addition of the enzyme Aii20J induced a generalized decrease in biofilm formation obtained from GCF samples of healthy donors and patients with periodontitis. These changes were not accompanied by a significant shift in bacterial communities, which is expected in this approach, as QSI does not affect bacterial growth (Parga et al., unpublished results). The addition of AHL analogs has also been explored as a strategy to study the effect of QSI in oral biofilms, with reports of reductions in biofilm formation in oral pathogens, such as *P. gingivalis* [171]. Naturally present QQ activities in human saliva samples could point towards a role of AHL-mediated QS in oral biofilms [136], but further confirmation of the relevance of these findings is needed. As seen with QSI approaches against AI-2 and AIP-mediated QS, these promising *in vitro* studies should be translated into *in vivo* and clinical trials to assess their potential. Some patents on QSI compounds for oral health maintenance have been filed [156], and some have been registered already [172]. Pathogenic bacteria are more susceptible to antibiotic therapy after treatment with QSI compounds, as assessed by different studies [173, 174], opening the possibility of using QSI compounds as adjuvants to treat biofilm-associated infections. Before these approaches can be proposed in the clinical practice, our knowledge of microbe-microbe interactions in multispecies biofilms and the influence of QS molecules in the equilibrium between commensal and potentially pathogenic bacteria in the mouth needs to be expanded.

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3. Conclusion

The complex and multifactorial nature of caries, periodontitis, and the emerging problem of peri-implantitis, presenting no single cause, invalidate the use of simplistic preventive solutions such as eliminating one type of organism or complete removal of the dental plaque [12]. Therefore, the future of oral disease management will be based on targeted approaches to restore the microbiome equilibrium [3] and increase the resilience of the resident homeostatic microbiota [3, 11–13]. These strategies will probably require a personalized diagnosis of the dysbiosis state of the patients.

Among the different ecological strategies that are being explored and have been reviewed here, special attention should be paid to those that seem to be already involved in maintaining the homeostasis of the healthy oral microbiota, such as bacteriophages, quorum sensing inhibitors, or specific antimicrobial peptides. These strategies have yielded promising results *in vitro*, but *in vivo* trials are mandatory before the potential of these novel ecological strategies targeting to maintain and restore the equilibrium of the oral microbiota can be confirmed.

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Figure Legends

Figure 1. Convergence of different bacterial metabolic pathways in the production of nitric oxide. Nitrate reduction can lead to nitric oxide generation (left). Arginine plays a role in pH homeostasis via the arginine deiminase (ADS) pathway (right), and in nitric oxide synthesis, via the nitric oxide synthase (NOS) pathway.

Figure 2. Quorum-sensing dependent regulation of gene expression of biofilm-related and virulence-related genes. Modified from Muras et al. [155].

In review

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The quorum quenching enzyme Aii20J modifies *in vitro* periodontal biofilm formation

Ana Parga[1], Andrea Muras[1#], Paz Otero-Casal[2,3], Alexandre Arredondo[4], Agnès Soler-Ollé[4], Gerard Alvarez[4], Luis D. Alcaraz[5], Alex Mira[6], Vanessa Blanc[4], Ana Otero[1]*

[1] Department of Microbiology and Parasitology, CIBUS-Faculty of Biology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain

[2] Department of Surgery and Medical-Surgical Specialties, Faculty of Medicine and Odontology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain

[3] Unit of Oral Health, CS Santa Comba-Negreira, SERGAS, Spain

[4] Department of Microbiology, Dentaid Research Center, Cerdanyola Del Vallès, Spain

[5] Department of Cellular Biology, Faculty of Science, National Autonomous University of Mexico, Coyoacán, Mexico

[6] Department of Genomics and Health, FISABIO Foundation, Valencia, Spain

*** Correspondence:**
Ana Otero
anamaria.otero@usc.es

[#] **Present address:**
Andrea Muras
Laboratory of Microbial Pathogenesis, Navarrabiomed, Public University of Navarre (UPNA), University Hospital of Navarra, IdisNa, Pamplona, Spain

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Abstract

Recent studies have revealed the presence of *N*-acyl-homoserine lactones (AHLs) quorum sensing (QS) signals in the oral environment. Yet, their role in oral biofilm development remains scarcely investigated. The use of quorum quenching (QQ) strategies targeting AHLs has been described as efficient for the control of pathogenic biofilms. Here, we evaluate the use of a highly active AHL-targeting QQ enzyme, Aii20J, to modulate oral biofilm formation *in vitro*.

The effect of the QQ enzyme was studied in *in vitro* multispecies biofilms generated from oral samples taken from healthy donors and patients with periodontal disease. Subgingival samples were used as inocula, aiming to select members of the microbiota of the periodontal pocket niche in the *in vitro* biofilms. Biofilm formation abilities and microbial composition were studied upon treating the biofilms with the QQ enzyme Aii20J.

The addition of the enzyme resulted in significant biofilm mass reductions in 30 – 60 % of the subgingival-derived biofilms, although standard AHLs could not be found in the supernatants of the cultured biofilms. Changes in biofilm mass were not accompanied by significant alterations of bacterial relative abundance at the genus level. The investigation of 125 oral supragingival metagenomes and a synthetic subgingival metagenome revealed a surprisingly high abundance and broad distribution of homologous of the AHL synthase HdtS and several protein families of AHL receptors, as well as an enormous presence of QQ enzymes, pointing to the existence of an intricate signaling network in oral biofilms that has been so far unreported, and should be further investigated.

Together, our findings support the use of Aii20J to modulate polymicrobial biofilm formation without changing the microbiome structure of the biofilm. Results in this study suggest that AHLs or AHL-like molecules affect oral biofilm formation, encouraging the application of QQ strategies for oral health improvement, and reinforcing the importance of personalized approaches to oral biofilm control.

Contribution to the field

Nowadays, oral disease control largely relies on the use of antimicrobials. These treatments are losing efficacy due to an increase in bacterial resistances, prompting the search for alternative, more targeted strategies to complement current approaches. Oral disease progression is closely related to biofilm development, and a key factor driving bacterial biofilm formation is cell-cell communication mechanisms, also termed Quorum Sensing (QS). Here, we have investigated the potential of Aii20J, a QQ enzyme highly active against *N*-acyl-homoserine lactones (AHLs), to inhibit polymicrobial oral biofilms originated from subgingival samples of healthy donors and patients with periodontitis. We describe a great efficacy of Aii20J reducing biomass without altering the bacterial composition of the biofilms, supporting the use of QQ strategies for oral health improvement. The variability of biofilm responses observed among patients indicates the importance of using different donors to evaluate oral biofilm inhibitory strategies as a preliminary step before clinical trials. Moreover, this study is first in reporting the abundance of QS-related sequences in oral metagenomes, revealing an extremely high abundance of sequences related to AHL biosynthetic pathways, suggesting a role of these signal molecules in the oral environment.

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1 Introduction

Most oral bacteria are embedded in a matrix of extracellular polymers of host and bacterial origin and attached to the tooth surface forming a biofilm, presenting altered phenotypes compared to planktonic cells (Donlan and Costerton, 2002; Marsh, 2006). The two major oral bacterial diseases, dental caries and periodontal disease (PD), are caused by changes in local environmental conditions that result in a loss of the ecological equilibrium in the oral microbiota, eventually leading to a pathogenic biofilm (Marsh, 1994, 2003; Belda-Ferre et al., 2012; Hajishengallis et al., 2012). Therefore, it is not a single pathogen but rather the entire community residing in the oral cavity and its functional activities that are responsible for the progression of these diseases (Duran-Pinedo and Frias-Lopez, 2015; Yost et al., 2015). In this context, interfering with biofilm formation processes through modulation of microbial interactions has been proposed as a relevant strategy for preventing and treating oral polymicrobial diseases (Simón-Soro and Mira, 2015).

Cell density-dependent gene-regulation processes, known as quorum sensing (QS), have been proposed as potential targets for developing strategies to inhibit oral biofilm formation (Muras et al., 2018b, 2020b; Muras and Otero, 2020). Among the three most studied QS signals, the presence of Gram-positive autoinducer peptides has been reported in various oral streptococci, while the universal autoinducer-2 (AI-2) has been found in both Gram-positive and Gram-negative oral pathogens (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Kolenbrander et al., 2006). The *N*-acyl-homoserine lactones (AHLs), the QS signals typically used by Gram-negative bacteria, have traditionally been considered non-relevant in the oral cavity due to the lack of success in detecting their production in pure cultures of oral pathogens in the past (Whittaker et al., 1996; Frias et al., 2001; Burgess et al., 2002; Jakubovics and Kolenbrander, 2010; Huang et al., 2011; Guo et al., 2014). Despite this, a diversity of AHLs has been found in saliva samples and extracted teeth, including C_8-HSL, C_{14}-HSL, and C_{18}-HSL (Kumari et al., 2006; Muras et al., 2020b). Additionally, AHL-producing bacterial strains have been isolated from dentine caries (Goh et al., 2014, 2016) and human tongue surface (Yin et al., 2012b, 2012a; Chen et al., 2013). Previous studies had observed that AHLs and AHL analogs modified protein expression in *Porphyromonas gingivalis* (Komiya-Ito et al., 2006; Asahi et al., 2010) and the production of OC_8-HSL has been described in pure cultures of this periodontopathogen (Muras et al., 2020b). Moreover, a recent study on the effect of exogenous AHLs on *in vitro* oral biofilms proved that certain AHLs affected bacterial lactic acid production and protease activity and that the addition of C_6-HSL shifted the microbial composition of biofilms towards a periodontal bacterial profile (Muras et al., 2020a). In addition, bacterial gene clusters predicted to be related to homoserine lactone biosynthesis have been described in the genomes of bacteria from caries and periodontitis patients (Aleti et al., 2019). Together, these results suggest that the established role of AHLs in the current paradigm of cell-to-cell communication in oral biofilms should be revised (Muras and Otero, 2020; Muras et al., 2020b, 2022). Confirmation of the role of AHLs as QS signals in oral biofilm formation would open a broad range of possibilities for the prevention and treatment of oral diseases.

Different strategies to interfere with QS systems are present in natural environments. The use of these strategies in both natural and *in vitro* conditions has been successful in decreasing biofilm mass in single-species (Sun et al., 2021) and multispecies biofilms (Jo et al., 2016; Huang et al., 2019; Schwab et al., 2019; Jeong et al., 2020; Muras et al., 2020b, 2022). The use of these strategies is less likely to promote antimicrobial resistance than antibiotics, as they do not interfere directly with bacterial growth (Romero et al., 2014, 2015; Mayer et al., 2015, 2018; García-Contreras et al., 2016; Guendouze et al., 2017; Muras et al., 2018b). The enzymatic inactivation of QS signals, also known as quorum quenching (QQ), has been thoroughly studied (Romero et al., 2015), especially enzymes

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capable of disrupting AHLs (Dong et al., 2001). Remarkably, the presence of QQ activity against AHLs has already been described in cultivable bacteria from saliva and dental plaque samples (Muras et al., 2020b). The use of Aii20J, a broad-range, highly active AHL-lactonase belonging to the metallo-beta-lactamase family (Mayer et al., 2015), has yielded interesting results for controlling several AHL-regulated phenotypes in Gram-negative pathogenic bacteria (Mayer et al., 2015, 2018, 2020) and reducing oral biofilm grown *in vitro* from saliva and in a mixed biofilm of six oral pathogens (Muras et al., 2020b).

In this study, we assessed the effect of Aii20J on oral polymicrobial biofilms generated *in vitro* using samples from healthy donors and patients with PD. Subgingival samples were chosen to inoculate the biofilms, as they are expected to be more representative of the periodontal microbiota than saliva samples. *In vitro* biofilms were characterized in terms of total biomass and microbial diversity achieved in the presence of the enzyme. Additionally, supragingival shotgun metagenomes were investigated for the presence of QS- and QQ-related sequences.

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2 Materials and Methods

2.1 Subject recruitment

An initial group of 17 patients (female/male: 11/6; mean age: 48 ± 13 years) and 35 additional patients (female/male: 19/16; 60 ± 15 years) were recruited from the University Dental Clinic of the Faculty of Medicine and Dentistry at the University of Santiago de Compostela between December 2019 and March 2020, and between June 2020 and May 2022, respectively. In the first group of patients (n = 17), saliva and subgingival samples were taken to optimize biofilm generation conditions (see section 2.5). In the second group of patients (n = 35), only subgingival samples were taken, as a result of the optimization done with the first group. Oral health status was assessed according to the proceedings from the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions (Papapanou et al., 2018; Tonetti et al., 2018) (Supplementary Tables S1 and S2). Antibiotic treatment up to one month prior to sampling was the exclusion criterium set for the study.

2.2 Ethics statement

The investigation protocol 2009/319 for patient recruitment and sample handling, modified in July 2017, was approved by the Ethical Committee of Clinical Investigations of Galicia (Xunta de Galicia). Written informed consent was obtained from all participants in this study.

2.3 Production and purification of the AHL-lactonase Aii20J

The QQ enzyme Aii20J, a lactonase enzyme obtained from the QQ marine bacterium *Tenacibaculum* 20J (Romero et al., 2011, 2014), was obtained as previously described (Mayer et al., 2018). Briefly, protein expression was induced by adding 1 mM Isopropyl-D-thiogalatopyranoside to a suspension of a recombinant *E. coli* BL21(DE3)pLysS containing Aii20J. The induced cells were lysed by sonication, and purification of Aii20J was achieved using the His GraviTrap affinity column protein purification kit (GE Healthcare). The remaining imidazole from the purification buffer solutions was removed by dialyzing the sample in D-tubes (MWCO 10 kDa) (Merck KGaA, Darmstadt, DE) in sterile Milli-Q water. When appropriate, purified Aii20J was added to the biofilms at a working concentration of 20 μg mL^{-1}, corresponding to ten times the minimum concentration of purified enzyme needed to degrade 10 μM of C_6-HSL in 24 h. The AHL-degradation capacity of purified Aii20J and remaining Aii20J activity in biofilms' supernatants was routinely verified using a chromogenic assay (see "Quorum quenching activity solid plate assays" section below).

2.4 Quorum quenching activity solid plate assays

The AHL degradation activity of Aii20J, both purified and after total incubation time in biofilm supernatants, was tested using solid plate assays. QQ activity in saliva samples was assessed using the same methodology. Assays were carried out using the AHL biosensor strains *Chromobacterium subtsugae* CV026 for short-chain AHLs (McClean et al., 1997; Harrison and Soby, 2020) and *C. violaceum* VIR07 for long-chain AHLs (Morohoshi et al., 2008). Samples were mixed with either C_6-HSL (10 μM) or C_{12}-HSL (10 μM) (Sigma-Aldrich, Merck). These AHLs are commonly used as representatives of short- and long-chain AHLs, respectively (Muras et al., 2020b). PBS pH 6.5 with the corresponding AHL (10 μM) was used as negative control (Muras et al., 2018b). The pH of the saliva samples was checked beforehand and acidified when it exceeded pH 7 to avoid the spontaneous opening of the lactone ring of the AHLs at high pH values (Yates et al., 2002). All mixtures were incubated for 24 h at 22 °C.

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166 The presence of the AHLs was detected by adding a mixture of an overnight culture of the
167 corresponding biosensor and soft Luria-Bertani (LB) (0.8% agar) in a 1:4 proportion on top of an LB
168 agar plate. Wells were manufactured onto the agar, and 100 µL of the sample were placed in each
169 well. Plates were incubated for 24 h at 30 °C. The lack of violet pigmentation, as opposed to the
170 violet halo observed around the negative controls, indicated QS inhibition. This assay allows
171 identifying in a rather precise manner if the QS inhibition is due to an enzymatic degradation or to
172 the presence of a QS inhibitor molecule. Enzymatic degradation is often displayed as a lack of
173 pigmented halo or as a halo of reduced diameter respect to the negative control. On the other hand,
174 QS inhibitors diffuse with the AHL added to the sample; the different titers of both components, with
175 the AHL normally present in a much higher titer, result in an inner non-pigmented halo surrounded
176 by an outer violet halo (Muras et al., 2018a). Furthermore, this assay allows differentiating between
177 QS inhibition (observation of growth-turbid halos) and cell growth inhibition (observation of
178 transparent halos) (Muras et al., 2018a).

179 **2.5 Sample collection, biofilm generation, and biofilm quantification**

180 The initial biofilm collection was derived from saliva and subgingival samples from seven healthy
181 donors (codes H1 – H7) and ten patients with PD (codes P1 – P10) (Supplementary Table S1) and
182 cultured immediately. Saliva samples were collected in sterile tubes and diluted 1:100 in each culture
183 media used (Fernandez y Mostajo et al., 2017; Muras et al., 2020a). Subgingival samples consisted of
184 gingival crevicular fluid (GCF) collected by inserting 16 sterile paper points (4 in each mouth
185 quadrant) in the gingival sulcus for 1 minute. In patients with PD, samples were taken from the
186 deepest periodontal pockets. Samples were transported in 1 mL of thioglycollate broth (Merck), from
187 which a 1:53 dilution with the corresponding medium was made (Mira et al., 2019). For this initial
188 collection, saliva and subgingival samples were inoculated in 1) Brain Heart Infusion (BHI)
189 (PanReac, Barcelona, ES) in aerobic conditions and 2) Schaedler medium supplemented with vitamin
190 K1 (0.1 mg L^{-1}) (SCH-K1) (Conda Pronadisa, Madrid, ES) in anaerobic conditions. The Aii20J
191 enzyme was added at the moment of the inoculation, at a final concentration of 20 µg mL^{-1}. Negative
192 controls received sterile Milli-Q water. The purified Aii20J (35 kDa in size) was filtered through a
193 Centricon with a molecular weight cut-off of 10 kDa (Merck) to remove the enzyme and use the
194 remaining soluble fraction as an additional control (Supplementary Figure S7). A heat-inactivated
195 Aii20J (90 min at 121 °C) that did not conserve AHL-degrading activity was also tested
196 (Supplementary Figure S7). Biofilms were grown for 24 h at 37 °C.

197 *In vitro* oral biofilms (n = 17) were generated using the culture media and conditions described above
198 and measured in parallel using two different methodologies. The first is a modification of the
199 Amsterdam Active Attachment model (AAA model) (Exterkate et al., 2010; Muras et al., 2020a).
200 Briefly, the lids of 12-well cell culture plates (VWR) were replaced by custom-made stainless-steel
201 lids onto which 12 silicone tubes were attached, allowing for the insertion of glass coverslips (18 ×
202 18 mm) (Menzel Gläser, Braunschweig, DE) to serve as substrata for biofilm formation. The glass
203 coverslips fit vertically into the culture plate wells, filled with 3 mL of the corresponding adjusted
204 inocula (Muras et al., 2020b), prompting the active attachment of bacterial cells onto their surface.
205 Aii20J was added at a final concentration of 20 µg mL^{-1}. The AAA model allows the refreshment of
206 culture media and treatments at 12 h by transferring the lid with the glass coverslips to a new plate.
207 When required, anaerobiosis was achieved using an anaerobiosis jar (OXOID, Basingstoke, GB) with
208 AnaeroGen 2.5L Atmosphere Generation System (OXOID). Biofilms were quantified by crystal
209 violet (CV) assay. After incubation, coverslips were allowed to dry in a fresh plate, and biofilms
210 were stained for 20 minutes with 2 mL of a 0.04% CV solution (v/v in distilled water) (Panreac). The
211 excess dye was removed by washing twice with distilled water. Before releasing the CV for

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quantification, pictures were taken to perform visual examinations of the biofilms. When two independent observers found control and Aii20J-treated biofilms to be visually distinguishable, the biofilms were considered to have macroscopical differences (Supplementary Figure S2). Finally, CV bound to the biofilms was diluted in 3 mL of a 33% acetic acid solution (v/v in distilled water) (Scharlab, Barcelona, ES). Sample absorbance was measured at 590 nm. All experiments were performed in triplicate.

The second methodology used was the xCELLigence Real Time Cell Analyzer (RTCA) System (ACEA, Biosciences Inc., San Diego, US) (Muras et al., 2018b, 2020b; Mira et al., 2019). E-plates 16 (ACEA, Biosciences Inc.) were inoculated with 100 µL of the corresponding culture medium and 80 µL of the saliva/subgingival samples. To lactonase-treated samples, 20 µL of Aii20J (to a final concentration of 20 µg mL^{-1}) were added, while 20 µL of sterile Milli-Q water were added to the control wells. An overlay of sterile paraffin was added when an anaerobic atmosphere was required. The xCELLigence measures changes in impedance as cells attach to the bottom of the E-plates, and the software computes cell index (CI) values that correspond to the amount of biofilm formed. All experiments were performed in triplicate.

A broader screening of culture conditions was then performed using subgingival samples (n = 35). Samples were collected following the previously described methodology and cultured in the AAA model. Culture conditions for four initial patients (H8, G1, P11, and P12) (Supplementary Table S2) were BHI in aerobiosis, BHI in anaerobiosis, SCH-K1 in anaerobiosis, and semi-defined McBain medium (McBain et al., 2005; Janus et al., 2015) in anaerobiosis. Subsequent biofilms from ten healthy donors (H9 – H18), two patients with gingivitis (G2, G3), and 19 patients with periodontitis (P13 – P31) (Supplementary Table S2) were inoculated in BHI-anaerobiosis and McBain-anaerobiosis. Biofilms were grown for 24 h at 37 °C, with culture media and treatment refreshment at 12 h. Biofilm quantification was performed as previously described.

2.6 Confocal-laser scanning microscopy

Subgingival-derived biofilms from patients P30 and P31 grown in McBain-anaerobiosis in the AAA model were further visualized using confocal-laser scanning microscopy (CLSM) to compare control and Aii20J-treated biofilms. Biofilms formed onto the glass coverslips were stained with two nucleic acid dyes; SYTO 9, which is membrane permeable and enters all bacterial cells, and propidium iodide (PI), which only enters membrane-damaged cells (LIVE/DEAD BacLight Bacterial Viability Kit, Thermo Fisher Scientific, Waltham, US). The staining solution was prepared by mixing SYTO 9 and PI in a 1:1 proportion and then diluting it 150-fold in sterile PBS (pH 7.4). Approximately 50 µL of the staining solution were poured onto each biofilm. Samples were kept for 15 minutes at room temperature and protected from light before image acquisition with a Leica Stellaris 8 FALCON confocal microscope, with the objective HC PL APO CS2 20x / 0.75 Dry (Leica Microsystems GmbH, Wetzlar, DE). Biofilms were observed using the 499 nm laser excitation and 504 – 555 nm emission band for SYTO 9, and 561 nm excitation laser and 570 – 675 nm emission band for PI. Photographs were acquired at 20x magnification. To calculate the area covered by biofilms, eight fields for each condition were randomly selected and processed using the Leica Application Suite X software (LAS X v.3.7.4, Leica Microsystems). The area emitting photons from the SYTO 9 and PI channels was divided by the total field of view. The results are expressed in percentage of the area covered by biofilm structures in each condition.

2.7 Harvesting of biofilms and DNA extraction

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Genomic DNA for sequencing analysis was obtained from biofilms grown in the AAA model. Biofilms were harvested by transferring the glass coverslips into 5 mL of sterile PBS. Then, they were sonicated for 15 minutes to disperse the biomass. Genomic DNA extraction was done using the "DNeasy PowerBiofilm Kit" (Qiagen, Germantown, US), following the manufacturer's instructions. Briefly, biofilms were subjected to chemical and mechanical lysis. Proteins and inhibitors were removed, followed by pH-driven precipitation of large insoluble macromolecules. Total genomic DNA was captured using a silica spin filter column and finally eluted. DNA concentration was measured using a NanoDrop (Thermo Scientific).

2.8 Library preparation and microbiome analysis

Microbial genomic DNA (5 ng μL^{-1} in 10 mM Tris pH 8.5) was used to amplify the 16S rRNA gene V3-V4 hypervariable regions. After size verification, the libraries were prepared according to the supplier's protocol (Illumina, Inc.), and the resulting 11 pM 20% on PhiX pooled library was sequenced using a 2x300 base pairs paired-end run (MiSeq Reagent kit V3(MS-102-3001)) on a MiSeq Sequencer.

Quality assessment was performed using the prinseq-lite program (Schmieder and Edwards, 2011). The pipeline DADA2 (Callahan et al., 2016) was used to analyze and cluster the sequences into amplicon sequence variants, classified to the genus level with the SILVA database (Quast et al., 2013). Computations and statistics were carried out in R RStatistics (R Core Team, 2022) using knitr, knitcitations, markdown (Allaire et al., 2014; Boettiger, 2014; Xie, 2014), biostrings, and vegan (Oksanen et al., 2017). The differential relative abundance of the bacterial genera identified was assessed with the packages Phyloseq and DESeq2 (Love et al., 2014). Differences were filtered with a log2 fold change threshold higher than 2 and by their base mean value, discarding the lower quartile. Beta diversity was studied using a PCoA of weighted UniFrac distances using the package Phyloseq. Differences between samples, grouped according to the studied variables, were analyzed using the PERMANOVA test implemented in the adonis function of the package vegan (Oksanen et al., 2017). Alpha diversity was analyzed with the R package Phyloseq (McMurdie and Holmes, 2013), using the Chao1 and the Shannon and Simpson indexes as richness and diversity estimators. The data's normality and homoscedasticity were assessed before choosing parametric or nonparametric tests included in the package stat (R Core Team, 2022).

2.9 Bacterial quantification by quantitative PCR

The amount of total bacteria, *P. gingivalis,* and *P. endodontalis* present in 33 subgingival biofilms from patients with periodontitis and grown in McBain-anaerobiosis in the AAA model was evaluated by quantitative PCR (qPCR) in a LightCycler 480 II Thermocycler (Roche Diagnostics GmbH, Mannheim, DE). The reactions were performed in a 20 µL volume containing LightCycler 480 II Probes Master (Roche Diagnostics GmbH), specific primers and probes for each bacteria or universal primers for Eubacteria (Supplementary Table S3), 5 µL of isolated DNA and PCR grade sterile water. Positive and negative controls and sample reactions were performed in duplicate. The samples underwent an initial amplification cycle of 95 °C – 10 min, followed by 40 cycles of 95 °C – 10 s, annealing at 60 °C – 30 s and extension at 72 °C – 1 s. Crossing points (C_P) were calculated with LightCycler 480 Software 1.5 (Roche Diagnostics GmbH) using the second derivative maximum method. Crossing point values were then extrapolated to colony-forming units (CFUs) using standard curves, as described in Àlvarez et al. (2013). Briefly, to obtain standard curves, first pure bacterial cultures were generated in three biological replicates. When cultures reached log phase, CFU plating and, in parallel, DNA extractions for qPCR amplification were performed, both in 10-fold serial dilutions. Then, curves correlating CFUs/mL and C_P values were generated using the qPCR software.

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Reproducibility of the R^2 values of the curves obtained from the different biological replicates was used as a quality criterium to consider the correlations reliable (Àlvarez et al., 2013).

2.10 AHL extraction and preparation for HPLC-MS analysis

To detect the presence of AHLs at low concentrations in untreated biofilms, the supernatants of the biofilm cultures were extracted to concentrate the polar molecules and analyze them by high-performance liquid chromatography-mass spectrometry (HPLC-MS). Glass coverslips from the AAA models were transferred, together with the spent culture media, into fresh tubes (VWR) and sonicated for 15 minutes to disperse the biomass. Samples were then acidified with HCl 1 M to induce the circularization of the lactone rings (Yates et al., 2002). Samples were extracted sequentially with two volumes of ethyl acetate (Scharlab) and two volumes of dichloromethane (Scharlab) (Muras et al., 2020a). The organic phases were evaporated in a Rotavapor-R (Büchi), and the dry samples were resuspended in 1 mL of acetonitrile (Merck) and stored at -20 °C until its quantification by HPLC-MS. Fresh culture media were also extracted following the same procedures.

HPLC-MS analyses were performed at the Unit of Chromatographic Techniques and Water Analysis (University of A Coruña, ES) using an HPLC 1100 series (Agilent). The mass spectrometer (MS) used was an API 4000 triple quadrupole (Applied Biosystems). A calibration curve was obtained running standard synthetic AHLs (with and without oxo- and hydroxy-substitutions, ranging from 4 to 18 carbons in their acyl chains). The calibration curve was used to quantify AHLs in the samples. Elution times and molecular spectra of the standard synthetic AHLs were used to identify the AHLs present in the samples (Romero et al., 2010; Mayer et al., 2018).

2.11 Search of quorum-related sequences in oral metagenomes

A selection of QS and QQ-related sequences was made by manually searching NCBI's databases (https://www.ncbi.nlm.nih.gov/) for annotated nucleotide and amino acid sequences with demonstrated activity. Selected QS sequences comprised AHL synthases (n = 36), AHL receptors (n = 28), the AI-2 synthase LuxS (n = 11), and AI-2 receptors (n = 3). PQS synthases (n = 2) and a PQS receptor (n = 1), were included as well. QS "dialect" systems were also selected, including non-AHL synthases (n = 5) and receptors (n = 10) in the analyses. Selected QQ sequences included three types of enzymes: lactonases (n = 63), acylases (n = 20), and paraoxonases (n = 3). Nucleotide sequences were translated into amino acid sequences using the software Expasy (Duvaud et al., 2021).

The initial 182 sequences were clustered into protein families using CD-HIT (Fu et al., 2012), establishing a minimum of 70% length coverage and 40% sequence identity to reduce redundancy. Even though several protein sequences have the same annotation, some were clustered into different protein families, reflecting evolutionary divergence, e.g., LuxS sequences were clustered into four groups with the representative type sequences corresponding to *Vibrio harveyi, Streptococcus pyogenes, Bacillus thuringiensis,* and *Bacteroides heparinolyticus*. The 182 sequences were clustered and grouped by function, resulting in 78 protein families. The accession numbers of the sequences in each protein family are available in Supplementary Table S4.

The representative sequences selected for each protein family were then used to query 118 shotgun oral metagenomes from the Human Microbiome Project (HMP) (Human Microbiome Project Consortium, 2012a, 2012b) (https://portal.hmpdacc.org/) (Supplementary Table S5) and seven oral metagenomes from a Spanish collection (Belda-Ferre et al., 2012). These 125 metagenomes corresponded to supragingival plaque samples. Due to the absence of metagenomic data from subgingival sites in the HMP, a "synthetic" subgingival metagenome was generated (Supplementary

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Table S6). To select the information contained in this metagenome, the 16S rRNA gene sequencing data from subgingival and supragingival sites available in the HMP (Human Microbiome Project Consortium, 2012b) was investigated. First, a comparison of the relative abundances of the bacterial genera present in subgingival and supragingival niches was done to evaluate the resemblance between both sites (Supplementary Table S7). Then, the bacterial genera of subgingival sites with abundance values over 1% were selected, and the genomes of the most representative species from each genera were retrieved (Supplementary Table S6) to generate the synthetic subgingival metagenome.

Sequence searches were performed using DIAMOND (Buchfink et al., 2021) with a minimum subject coverage of 70% and an e-value cut-off of 1e-10. Alignment results were parsed into tables using in-home scripts (available at https://github.com/genomica-fciencias-unam/tabla_genes). Statistical analyses were done using R (R Core Team, 2022) with the phyloseq (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2009), vegan (Oksanen et al., 2017), and tydiverse (Wickham et al., 2019) packages. All R procedures are available as a jupyter notebook in the Supplementary Material.

2.12 Statistical analyses

Statistical analyses were performed using Prism 8.3.0 (GraphPad, San Diego, CA, USA, www.graphpad.com) or R (R Core Team, 2022). Two-tailed Student's *t*-tests (referred to in the text as *t*-tests) or ANOVAs were performed for normally distributed samples. Mann-Whitney tests were performed for non-normally distributed samples to compare control and Aii20J-treated biofilm mass and cell index values within the same donor. Wilcoxon matched-pairs signed-rank tests (referred to in the text as Wilcoxon tests) were performed to assess the significance of the differences between biofilms that were reduced in the presence of the enzyme and those that were increased in the presence of the enzyme, subject by subject, grouped according to their oral health status. Quantitative PCR results were analyzed using Mann-Whitney tests for independent samples. For all the statistical analyses, significant differences were determined with an $\alpha = 0.05$.

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3 Results

3.1 Effect of sample origin and cultivation systems on *in vitro* oral biofilm response to the AHL-lactonase Aii20J

To assess the potential use of Aii20J in the prevention of oral diseases, the biofilm inhibitory capacity of the enzyme was first explored using two cultivation techniques: the AAA model (Exterkate et al., 2010; Muras et al., 2020a) and the xCELLigence system (ACEA, Biosciences Inc.). The AAA model was assembled with vertically placed glass coverslips onto which bacterial cells can only attach in an active manner. The AAA model allows the refreshment of culture media, further prompting biofilm formation. Besides, the substrata can be easily manipulated at the end of the incubation time to perform quantification assays, microscopy observations, and DNA extraction, allowing a complete characterization of the biofilms that is hampered by the use of the CV assay alone (Muras et al., 2018b). On the other hand, the xCELLigence system is a real-time monitoring technique based on cell index (CI) values that correlate to the amount of biofilm formed onto the bottom of modified culture plates (Mira et al., 2019). This system has been used to monitor oral biofilm formation (Mira et al., 2019), but is not suitable to detect structural differences in biofilms. Both systems were inoculated with saliva and subgingival samples from 17 subjects (Supplementary Table S1). Samples were grown aerobically in BHI medium and anaerobically in SCH-K1. Both culture media contain a high protein load, but the Schaedler medium has additional components that favor the growth of anaerobes, such as cysteine and hemin, and in addition to the commercial recipe, vitamin K1 (Murray, 1978; Marsh, 2016). The use of either aerobic or anaerobic atmospheres aimed to assess whether the effect of the enzyme would be different on bacterial communities grown under such conditions. We observed important variations in biofilm formation capacity among samples (Supplementary Figures S3 and S4) without significant differences in biomass values between healthy and PD groups grown in the AAA model (*t*-test) (Supplementary Figure S3). In the xCELLigence system, PD samples yielded significantly higher cell index values than healthy samples for subgingival biofilms grown in BHI and saliva biofilms grown in SCH-K1 (*t*-test) (Supplementary Figure S4A,D). In addition, initial biofilm mass achieved in the control condition did not correlate with sensitivity to Aii20J in any cultivation system (Pearson r) (data not shown). Regarding the effect of Aii20J on biofilm formation, when cultivated in the AAA model, subgingival biofilms were more sensitive to the enzyme than saliva biofilms in both culture media (Figure 1). Analysis of the effect of the enzyme on subgingival biofilms grouped according to the oral health status of the patients showed a significant decrease in PD-derived biofilms grown in BHI and both healthy- and PD-derived biofilms grown in SCH-K1 (Wilcoxon test) (Figure 1A,B). For saliva biofilms cultured in the same conditions in the AAA model, only PD samples grown in SCH-K1 presented a significant biomass decrease (Wilcoxon test) (Figure 1C,D). However, the xCELLigence system was much less sensitive than the AAA model in detecting the effect of Aii20J, as significant changes were only found in two saliva-derived biofilms treated with Aii20J, either in BHI or SCH-K1 (*t*-test) (Supplementary Figure S2). Therefore, the AAA biofilm cultivation model and subgingival samples were selected for further experiments.

Additionally, we evaluated the presence of QS inhibitory activity among the initial saliva samples against short- (C_6-HSL) and long-chain (C_{12}-HSL) AHLs with standardized bioassays (Romero et al., 2011). QQ activity against C_{12}-HSL was common, with five out of seven saliva samples from healthy donors and seven out of eight saliva samples of PD origin inhibiting AHL detection by the biosensor strain VIR07 (Supplementary Figure S5). Conversely, QQ against C_6-HSL was scarce, with only two healthy and one PD saliva samples decreasing violacein production in the biosensor strain CV026

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(Supplementary Figure S5). The small volume of the saliva samples collected did not allow assessing the presence of AHLs in these samples.

To assess which culture media and incubation conditions resulted in higher microbial diversity within the PD-derived biofilms, we chose four culture conditions: BHI-aerobiosis, BHI-anaerobiosis, SCH-K1-anaerobiosis, and in addition, McBain-anaerobiosis (Figure 2A,B). The use of McBain culture medium provides with a high protein load, and like the SCH-K1 medium, McBain comprises cysteine, hemin, and vitamin K1, that favor the proliferation of Gram-negative anaerobes. To achieve a higher resemblance with the oral environment, the McBain medium contains also mucin, one of the major salivary polypeptides, present in the acquired enamel pellicle. Experiments were performed using subgingival samples, as they are expected to be more representative of the periodontal microbiota than saliva samples. Regarding microbial diversity, the genus *Streptococcus* was predominant in all biofilms (Figure 2B), but McBain medium harbored the most diverse biofilms, with increased proportions of genera such as *Veillonella, Granulicatella, Gemella,* and *Haemophilus* both in control and Aii20J-treated biofilms, as compared with the other culture media. The presence of the QQ enzyme resulted in significant reductions of subgingival-derived biofilm mass even in Gram-positive predominated biofilms obtained in BHI and SCH-K1 media, without significantly changing bacterial relative abundance at the genus level (Figure 2A,B).

3.2 Effect of the AHL-lactonase Aii20J on *in vitro* subgingival-derived biofilm formation and bacterial diversity

Once we established the optimal conditions for obtaining diverse oral biofilms in the AAA model, we studied a greater number of samples, assessing changes in biofilm mass and microbial diversity in the presence of Aii20J. Subgingival samples were chosen as inocula since they present higher sensitivity to Aii20J than saliva samples, as seen in previous experiments (Figure 1). For these experiments, 35 patients were recruited; 11 healthy donors, three patients with gingivitis, and 21 with periodontitis (Supplementary Table S2). Biofilms were grown in BHI-anaerobiosis and McBain-anaerobiosis to compare the effect of Aii20J on biofilms dominated by Gram-positive bacteria and in more diverse biofilm communities, respectively. As seen with the initial set of patients, biofilm formation abilities varied widely depending on the donor (Supplementary Figure S6), with no significant differences in biomass values between healthy and PD groups being observed (*t*-test) (Supplementary Figure S6). Biomass values of untreated biofilms did not correlate with sensitivity to Aii20J (Pearson r) (data not shown).

Biomass was reduced in the presence of Aii20J in biofilms grown in both BHI-anaerobiosis and McBain-anaerobiosis, with few exceptions (Figure 3), confirming previous results obtained in *Streptococcus*-dominated biofilms (Figure 1). When the effect of Aii20J was analyzed by grouping the biofilms according to the oral health classification of the patients, PD-derived biofilms were significantly reduced in all culture conditions, whereas healthy-derived biofilms were significantly reduced only when grown in BHI (Wilcoxon test) (Figure 3). In subgingival, PD-derived biofilms, macroscopical differences (Supplementary Figure S1) were detected in 71% of biofilms grown in BHI and 38% of biofilms grown in McBain in the presence of Aii20J, and biomass decrease was statistically significant in 50% and 42% of the samples, respectively (*t*-test) (Figure 3). In subgingival, healthy-derived biofilms, macroscopical differences were found in 91% of biofilms grown in BHI and 18% of biofilms grown in McBain. Additionally, biomass reduction due to Aii20J was significant in 64% of healthy biofilms grown in BHI and 18% of healthy biofilms grown in McBain (*t*-test) (Figure 3). Assessment of additional controls, i.e., the fraction smaller than 10 kDa resulting from the purified Aii20J solution and the heat-inactivated Aii20J, yielded no differences in

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biofilm mass compared with water-only negative controls in biofilms from two periodontal donors susceptible to Aii20J (Supplementary Figure S7).

Subgingival biofilms from patients P30 and P31 grown in McBain were visualized under CLSM to compare biofilms that presented different responses to Aii20J in the CV assay (Figure 3B). Biofilms from patient P30 were significantly reduced in biomass in the CV assay and also showed a significant reduction in biofilm coverage when comparing CLSM photographs of control (11.0 ± 4.9%) and Aii20J-treated biofilms (5.8 ± 3.7%) (*t*-test) (Supplementary Figure S8). On the other hand, biofilms from patient P31 presented no significant differences in biofilm mass in the CV assay nor in biofilm coverage between control (3.26 ± 2.57%) and Aii20J-treated biofilms (3.57 ± 1.43%) (Supplementary Figure S8). However, control biofilms of patient P31 were predominantly stained with PI, whereas Aii20J-treated biofilms were predominantly stained with SYTO 9 (Supplementary Figure S8).

In parallel to biofilm quantification, biofilm mass from control and Aii20J-treated samples grown in McBain was collected for DNA extraction, as we previously observed that this culture medium allowed higher microbial diversity in biofilms from subgingival samples (Figure 2). Sequencing of the 16S rRNA gene confirmed the dominance of the genus *Streptococcus,* reaching a 45 – 61% relative abundance in the biofilms (Figure 4A,B,C). *Veillonella, Haemophilus, Granulicatella, Gemella,* and *Aggregatibacter* were also found in all biofilms within the top ten most relatively abundant genera. Beta diversity examined with Principal Coordinate Analysis (PCoA) and alpha diversity indexes revealed no significant differences in microbial composition between control and Aii20J-treated biofilms (Figure 4D,E,F, Supplementary Figure S9). In addition to diversity indexes, a differential abundance analysis was performed grouping the biofilms according to the oral health status of the donors. In periodontitis-derived biofilms, the genus *Porphyromonas,* among others, significantly decreased its differential abundance in the presence of Aii20J (Figure 5A). Further quantification of *Porphyromonas gingivalis* and *Porphyromonas endodontalis*, both species linked to oral diseases (Hajishengallis et al., 2012; de Brito et al., 2020), was done using qPCR on periodontitis-derived biofilms (n = 19). No significant differences were found for *P. gingivalis* nor *P. endodontalis* CFUs between control and Aii20J-treated biofilms (Mann-Whitney test) (Figure 5B). Total 16S rRNA gene quantification for Eubacteria was performed as a normalization control.

Last, supernatants of untreated biofilms (n = 16) were investigated for the presence of the most common AHLs (from C_4-HSL to C_{18}-HSL, either with or without oxo and hydroxy substituents) by HPLC-MS analysis. None of these standard AHLs were identified in any of the samples.

3.3 Abundance of quorum-related sequences in oral supragingival metagenomes

The absence of standard AHLs in the supernatants of biofilm cultures and the impossibility of performing the same analytical procedures to detect AHLs on the original saliva and subgingival samples due to their reduced volume prompted the investigation of the presence of sequences related to AHL synthesis and sensing, as well as AHL enzymatic degradation in 125 metagenomes from supragingival plaque samples available in public databases (Belda-Ferre et al., 2012; Human Microbiome Project Consortium, 2012a, 2012b), and one synthetic subgingival metagenome that comprised the most representative bacterial species from the subgingival niche (Supplementary Table S6), and that displayed very similar results to those of the supragingival metagenomes in terms of sequences found and their abundance. The analysis of these metagenomes revealed a surprisingly high abundance of proteins homologous to the AHL synthase HdtS (Figure 6, Supplementary Table S4). This protein family comprised three HdtS sequences from *Pseudomonas,* and it appeared in

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503 nearly every metagenome, almost as abundantly as the four protein families of LuxS, included as
504 reference in this analysis (Figure 6). Among the AHL receptors investigated, three protein families
505 appeared in elevated abundance in both the supragingival metagenomes and the synthetic subgingival
506 metagenome. Representative members of these families are AbaR of *Acinetobacter,* RhlR of
507 *Pseudomonas,* and the promiscuous AHL-receptor SdiA of *Burkholderia* (Figure 6). These protein
508 families appeared present in a high number of metagenomes, although their abundance was lower
509 than that observed for HdtS. It is worth mentioning that the protein family represented by RhlR also
510 comprised several SdiA sequences, including those from *Escherichia coli* and *Salmonella,* that were
511 divergent from those classified together with the SdiA sequences of the *Burkholderia* family.

512 In addition to AHL synthases and receptors, we investigated the supragingival and subgingival
513 metagenomes for the presence of AHL-inactivating enzymes: lactonases, acylases, and paraxonases.
514 Protein families of lactonase and acylase enzymes appeared in extremely high abundances, in some
515 cases, even higher than LuxS sequences (Figure 6). These *in silico* results align with those obtained
516 *in vitro* on the high abundance of QS inhibitory activity among saliva samples (Supplementary
517 Figure S5).

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4 Discussion

This work aimed to evaluate the potential of the QQ enzyme Aii20J to prevent biofilm formation of periodontal origin based on *in vitro* studies. Given the rise of antimicrobial resistance in dentistry, alternative approaches to control periodontal diseases need to be explored (Cieplik et al., 2014; Rams et al., 2014). Several studies have described the effectiveness of QQ strategies against monospecific (Sun et al., 2021) and polymicrobial biofilms (Jo et al., 2016; Huang et al., 2019; Schwab et al., 2019; Jeong et al., 2020). Specifically, the inhibitory effect of the AHL-lactonase Aii20J on biofilm formation obtained from saliva samples has already been described (Muras et al., 2020b). Aii20J is a wide-spectrum, extremely thermoresistant enzyme that has no genotoxicity (UNE-EN ISO 10993-3:2015) and no cytotoxicity in eukaryotic cells (OECD Guideline 471) (unpublished results). Besides, we have confirmed that Aii20J retains its activity after 24 months in a commercial mouthwash at room temperature (unpublished results). Despite being a member of the metallo-beta-lactamase super-family, Aii20J does not affect beta-lactam antibiotics (Mayer et al., 2015) nor interferes with bacterial growth (Muras et al., 2018b), reducing the risk of inducing antimicrobial resistance. These properties make Aii20J a promising candidate for developing antibiofilm strategies in the oral health context.

Our results indicate that the AAA model, together with CV staining, is a more sensitive methodology than the xCELLigence system for detecting different responses of biofilms to Aii20J (Figure 1). The AAA model allows quantification, macroscopic evaluation (Supplementary Figure S1), and microscopy imaging (Supplementary Figure S8) of the biofilms attached to the glass coverslips, complementing the CV assay for detecting structural differences (Muras et al., 2018b). The AAA model also facilitates the refreshment of treatments and culture media, making more nutrients available for the attached cells, eliminating loose bacteria and metabolic waste, and ensuring the assessment of actively attached cells only. On the other hand, the xCELLigence system monitors biofilm growth during early adhesion stages (Muras et al., 2018b; Mira et al., 2019), reaching saturation after 10 h of incubation (Muras et al., 2018b). In the AAA model, an end-point quantification technique, we describe significant biofilm alterations due to Aii20J. In contrast, in the xCELLigence system, a real-time monitoring technology, no significant changes were found for most samples. Thus, Aii20J may affect biofilm in its maturing phase rather than in the initial attachment stages.

Different culture media and conditions were examined to achieve biofilms that contained higher proportions of anaerobic Gram-negative bacteria. Biofilms inoculated in BHI and SCH-K1 harbored populations dominated by the genus *Streptococcus* (Figures 2 and 4). However, McBain medium, containing a higher protein load than the other culture media, allowed the proliferation of more diverse communities (Figure 4). These conditions (the use of McBain in anaerobiosis) have already been proposed as an adequate option to recreate the gingival crevice niche *in vitro* (Janus et al., 2015), as high availability of nutrients and low oxygen conditions have been described to favor the proliferation of asaccharolytic and proteolytic bacteria *in vivo* (Drucker, 2000).

The addition of Aii20J resulted in significant biofilm mass decreases in most conditions tested. In the initial set of biofilms grown in BHI-aerobiosis and SCH-K1-anaerobiosis from different inocula, up to 41% of subgingival-derived biofilms were significantly reduced compared with the 29% of significantly reduced saliva biofilms (Figure 1). This differential response of the biofilms to the enzyme could be explained by the variation of bacterial communities between saliva and subgingival samples (Simón-Soro et al., 2013). We also describe an increased sensitivity to Aii20J in PD samples compared to healthy samples, finding significant biofilm reductions in PD-derived biofilms treated

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563 with Aii20J in three of four conditions tested (Figure 1). Differences in the behavior of healthy- and
564 PD-derived biofilms to the enzyme could be attributed to differences in genera that appear at low
565 relative abundance. In subsequent experiments, subgingival samples from healthy and PD patients
566 were grown under conditions driving to more diverse biofilms. Once more, Aii20J caused significant
567 reductions in biomass in both diverse biofilms (McBain-anaerobiosis), and biofilms dominated by
568 Gram-positive taxa (BHI-anaerobiosis), with 35 – 87% of samples presenting macroscopical
569 differences, respectively (Figure 3). These results further confirm the effectiveness of this enzyme as
570 an *in vitro* biofilm inhibitory strategy. Even in non-responsive biofilms, as for patient P31, we could
571 observe an effect of the enzyme on the biofilms using CLSM (Supplementary Figure S8).
572 Specifically, untreated biofilms from patient P31 were predominantly stained with PI, i.e., contained
573 mostly membrane-damaged cells, whereas Aii20J-treated biofilms were predominantly stained with
574 SYTO 9, indicating that most cells were membrane-intact (Supplementary Figure S8). Reductions in
575 response to Aii20J ranged from 50 – 99% of initial biomass in several biofilms and between 20 –
576 50% of initial biomass in most biofilms, whereas a small proportion presented little or no reductions
577 when treated with Aii20J. This contrasting behavior among biofilms generated from different
578 patients has already been described with the antibiotic amoxicillin (Mira et al., 2019). The observed
579 heterogeneity in biofilm formation abilities and response to the QQ enzyme indicates the importance
580 of using samples from different patients for assessing any oral treatment, as it allows observing
581 different responses among individuals when considering the administration of a treatment. This is of
582 special importance in the current context of oral health, with a growing consensus towards
583 personalized therapy and the proposals of studying individual responses on complex samples before
584 treatment administration (Bartold, 2017; Belibasakis et al., 2019).

585 Further investigations on the microbial diversity of biofilms grown in McBain revealed no significant
586 changes in microbial relative abundance between control and Aii20J-treated samples (Figure 4,
587 Supplementary Figure S9). In fact, similar studies on the effect of lactonases on polymicrobial
588 biofilm communities also reported that relative abundances in the microbial population were not
589 changed in the presence of QQ agents (Jo et al., 2016; Schwab et al., 2019). Conversely, some works
590 did observe changes in the relative abundance of oral- (Muras et al., 2020b) and water-associated
591 communities (Huang et al., 2019) in the presence of lactonases. The study of Muras et al. (2020b),
592 relied on the use of saliva samples from two subjects, whereas the present study increased to 33 the
593 number of subjects studied, and used subgingival samples instead of saliva for investigating changes
594 in microbial populations under the influence of Aii20J. These differences can explain the contrast in
595 the findings reported by both studies. Additionally, Muras et al. (2020b) described changes in
596 bacterial population only at the species level, as 99.74% of the population belonged to the genus
597 *Streptococcus*. In contrast, in the present study, biofilm populations comprised *Streptococcus*
598 proportions of 45 – 61%, and the assessment of changes in bacterial population was done at the genus
599 level, which can also explain the differences in the results of both studies.

600 Surprisingly, and despite the clear inhibitory effect observed in the presence of the Aii20J lactonase,
601 none of the most common AHLs were detected in the supernatants of untreated biofilm cultures in
602 this study. However, AHLs such as C_8-HSL have been detected *ex vivo* in extracted teeth, and C_8-
603 HSL, C_{14}-HSL, and C_{18}-HSL have been found *in vivo* in saliva samples (Muras et al., 2020b). In
604 addition, several oral isolates have been demonstrated to produce AHLs in pure or controlled mixed
605 cultures *in vitro* (Kumari et al., 2006; Yin et al., 2012b, 2012a; Chen et al., 2013; Goh et al., 2014,
606 2016; Muras et al., 2020b), and predicted AHL-related genes have been described in oral genomes
607 (Aleti et al., 2019). Here, culture conditions could be affecting the production of AHLs within the *in*
608 *vitro* biofilms, either as a consequence of the selection of non-AHL producer bacteria or as a result of
609 culture conditions affecting AHL producers. The latter has been previously demonstrated in the

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human pathogen *Acinetobacter,* which switches on and off its AHL-producing systems depending on incubation conditions *in vitro* (Mayer et al., 2018). Moreover, the low volume of biofilm and culture media available for AHL extraction could have hampered their detection by HPLC-MS, and we cannot fully rule out the presence of AHLs acting at a micro-scale within the biofilms.

The analysis of 125 supragingival plaque metagenomes and an additional synthetic subgingival metagenome revealed a high abundance of proteins related to AHL biosynthetic pathways. The abundance of LuxS was used as a reference, as it is implicated in central metabolism and, therefore, widely represented in Gram-positive and Gram-negative bacteria. However, AI-2, the molecule resulting from the detoxification of S-adenosylmethionine by the action of LuxS, can also act as a QS signal in some species (Schauder et al., 2001; Winzer et al., 2002). The fact that the response regulator of AI-2, the LuxR protein of *V. harveyi* (Showalter et al., 1990), is present in lower abundance in the metagenomes investigated (protein family "AI−2−Receptors−1−LsrR") (Figure 6), indicates a primary metabolic role of LuxS in these samples. In this study, we describe a remarkably high abundance of sequences related to HdtS synthases in both supragingival metagenomes and the synthetic subgingival metagenome (Figure 6). HdtS does not belong to the main AHL synthase families, and it has been demonstrated to produce several AHLs, including $OHC_{14:1}$-HSL, an uncommon AHL that can act as a bacteriocin (Laue et al., 2000). Besides, the analysis of the supragingival metagenomes revealed three protein families of AHL receptors present in high abundance levels among the metagenomes (Figure 6). Two of these families comprised several sequences of SdiA. SdiA is described as a LuxR solo, i.e., a bacterial receptor that cannot produce AHLs but respond to them (Michael et al., 2001). SdiA has been demonstrated to be highly promiscuous, presenting a broad ligand-binding specificity and being able to respond to molecules other than AHLs (Nguyen et al., 2015; Styles and Blackwell, 2018). Hence, we cannot fully disregard the possibility of signal molecules different from AHLs being the cognate ligands of SdiA receptors and being present in the oral environment. The increasing volume of studies on LuxR solos has revealed that these receptors can bind a wide range of AHL derivatives and molecules containing aromatic groups derived from fatty acid metabolic pathways (Tobias et al., 2020). These observations subscribe to those of Aleti et al. (2019), who found putative AHL-biosynthesis genes in the genomes of human oral taxa. Altogether, these results suggest a role of uncommon AHLs or yet-undescribed AHL-like molecules in oral biofilms that are not identified when using standard AHLs as reference but that could be sensed by broad-range specificity receptors, such as SdiA and other LuxR solos. The findings reported herein with the use of supragingival metagenomes constitute novel knowledge on AHL-related metabolism in the oral cavity. A first approach to assess the abundance of these sequences in bacteria from the subgingival niche has been done with a synthetic subgingival metagenome. With the increase in the number of metagenomic studies and publicly available metagenomic data, further investigations will help confirm the abundance of QS-related sequences in the oral cavity, specifically in the subgingival niche.

Last, the search for sequences related to AHL-quenching also yielded surprising results, with a widespread abundance of QQ enzymes in the supragingival metagenomes and the subgingival synthetic metagenome investigated (Figure 6). Finding the protein family "QQ-lactonases-14-AhlK" among the most abundant groups is particularly interesting (Figure 6). This protein family contains, among others, the enzyme AiiB from *Agrobacterium tumefaciens*, a lactonase with a broad specificity of substrates (Liu et al., 2007). The existence of several QQ enzymes capable of degrading AHL derivatives and even molecules other than AHLs has been described in the literature (Hiblot et al., 2013; Bergonzi et al., 2019). The high abundance in supra- and subgingival metagenomes of QQ enzymes that may be degrading other molecules apart from AHLs aligns with the finding of promiscuous receptors in those same metagenomes (Figure 6), strengthening the idea of the presence

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of AHL-like molecules in the oral cavity. Therefore, we hypothesize that the Aii20J-mediated biofilm inhibitory effect described here may not be derived from the action of Aii20J on AHLs but on other signal molecules of related structure. A similar case has already been described with the QQ lactonase SsoPox, whose antibiofilm activity against *Pseudomonas aeruginosa* monospecies biofilm is more dependent on its promiscuous activity on the *P. aeruginosa* PQS signal than on its activity against AHLs (Rémy et al., 2020). When Rémy et al. (2020) compared SsoPox with a lactonase without activity against PQS, the latter presented a much lower antibiofilm activity despite being more active against AHLs, suggesting that wider-range lactonases have greater antibiofilm properties. Hence, our results on the antibiofilm activity of the lactonase Aii20J on oral samples could be explained by a possible susceptibility of uncommon AHLs or AHL-like molecules to the enzymatic activity of Aii20J. This is further reinforced by the finding of the PQS synthase (protein family "PQS-Synthase-PqsABC"), in high abundance in the supragingival and subgingival metagenomes (Figure 6). The confirmation of the presence of such molecules would imply a more complex signaling network in oral biofilms than currently established. Furthermore, the effect of Aii20J on biofilms dominated by *Streptococcus*, a Gram-positive that is neither expected to respond to AHLs nor is affected by this QQ enzyme in *in vitro* pure cultures (Muras et al., 2018b), could be partially explained by the introduction of non-AHL signals in the scenery. Future investigations on the substrate specificity of Aii20J towards molecules other than standard AHLs will help validate this hypothesis.

We describe the use of QQ strategies, specifically of the QQ lactonase Aii20J, as successful in reducing multispecies biofilms *in vitro.* We propose the use of Aii20J as a modulatory strategy for oral disease control, as it results in lower bacterial load within the biofilm. The biofilms generated in the presence of the enzyme are expected to respond better to other treatments, such as antibiotic therapy, due to their reduced structural density, and their potentially increased porosity, presenting QQ strategies as possible coadyuvants in these therapies. Despite these promising results, further *in vitro* studies, including the evaluation of the effect of Aii20J at a transcriptional and proteomic level are required to understand the mechanisms underneath the behavioral changes detected in such biofilms. Additionally, changes in enzymatic activities within these polymicrobial communities must be assessed, as functional activities such as proteases or siderophore production are intimately linked to bacterial virulence. Previous studies have already described changes in enzymatic activities driven by the presence of AHLs in *in vitro* oral biofilms (Muras et al., 2020a), suggesting that similar changes could be observed with the use of AHL-degrading enzymes. Regarding the abundance of QS-related sequences in oral metagenomes, since all available metagenomes were of supragingival origin, the investigation of subgingival samples for the presence of AHL-related sequences would shed light on the extent of their distribution in the oral cavity, confirming a correlation with the periodontal diseases and endorsing the use of strategies targeting QS components for oral health care. The final objective of testing QS-targeting strategies would be their transfer from *in vitro* experiments to *in vivo* and clinical trials. To do so, it would be interesting to assess the possible synergies of Aii20J with antimicrobials. Also, it would be necessary to assess the optimal *in vivo* delivery doses, retaining time, and other pharmacological aspects when considering the addition of the QQ enzyme to oral products.

Findings in this work suggest an important role of AHLs or AHL-like molecules in oral biofilm formation. Targeting these molecules results in significant biomass reductions *in vitro* with no significant alterations in microbial relative abundance. This study is first in describing a remarkably high abundance of an AHL synthase and promiscuous AHL receptors on supragingival and subgingival metagenomes. Overall, these results indicate that QQ strategies could be useful for

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703 controlling oral biofilm formation while highlighting the importance of a personalized assessment in
704 the clinical practice.

705

706 **5 Abbreviations**

707 AAA model: Amsterdam Active Attachment model.
708 AE: aerobiosis.
709 AHLs: *N*-acyl-homoserine lactones.
710 ANE: anaerobiosis.
711 BHI: Brain Heart Infusion.
712 CFUs: colony-forming units.
713 CI: Cell Index.
714 C_P: Crossing points.
715 CSLM : confocal-laser scanning microscopy.
716 CV: crystal violet.
717 GCF: gingival crevicular fluid.
718 HMP: Human Microbiome Project.
719 HPLC-MS: high-performance liquid chromatography-mass spectrometry.
720 HSL: homoserine lactone.
721 IQR: interquartile range.
722 LB: Luria-Bertani.
723 MWCO: molecular weight cut-off.
724 PBS: phosphate-buffered saline.
725 PCA: Principal Components analysis.
726 PCoA: Principal Coordinates analysis.
727 PD: periodontal disease.
728 PI: propidium iodide.
729 qPCR: quantitative polymerase chain reaction.
730 QQ: quorum quenching.
731 QS: quorum sensing.
732 SCH-K1: Schaedler medium supplemented with 0.1 mg L^{-1} of vitamin K1.

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6 Conflict of Interest

The Aii20J enzyme used in this study is protected by the following patent: Otero, A., Romero, M., and Mayer, C. (2016). Peptide with quorum-sensing inhibitory activity, polynucleotide that encodes said peptide, and the uses thereof. PCT/ES2014/070569. The remaining authors declare not to have any conflict of interest.

7 Author Contributions

All authors contributed extensively to the work presented in this paper. AP and AMu performed experiments, analyzed data, and wrote the manuscript with contributions from AA, AS-O, GA, and LDA. PO-C recruited the patients, collected the samples, and provided each patient's oral health status classification. AMi helped in experiment planification and formal analysis. VB gave technical support and conceptual advice. AO conceived the project, designed experiments with contributions from VB, corrected the manuscript, and supervised the study. All authors discussed the results and implications and commented on the manuscript at all stages.

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11 Figures and figure captions

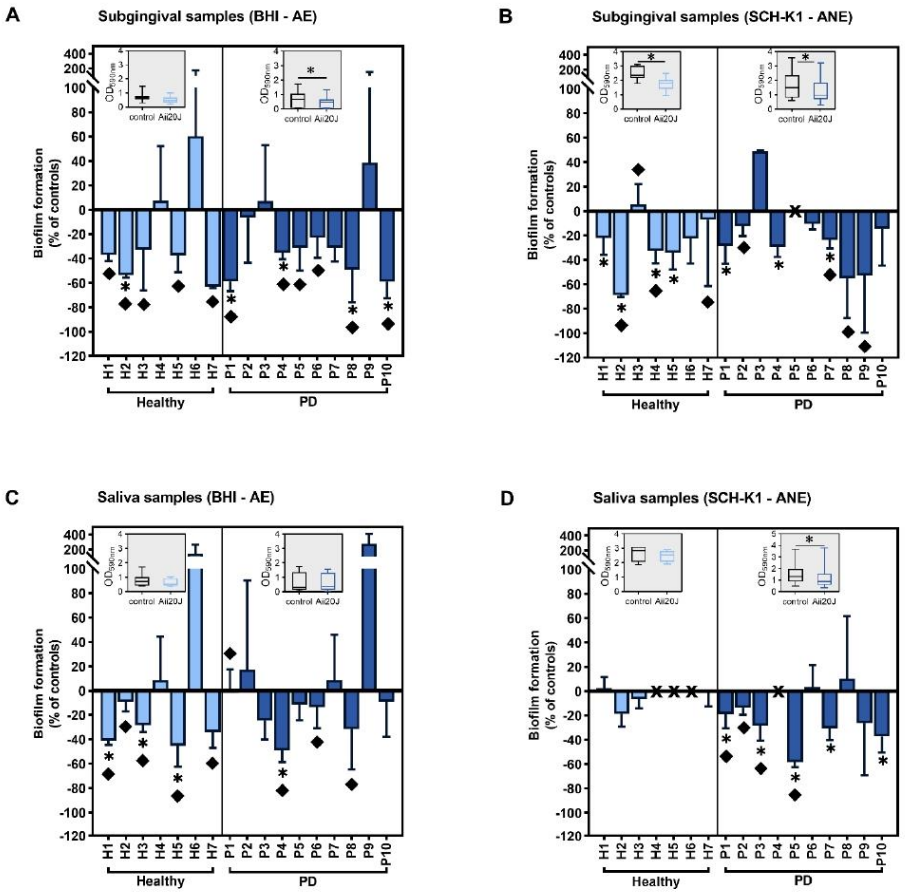


Figure 1. Inhibition of saliva and subgingival biofilm formation in the AAA model in the presence of Aii20J. Results are represented as mean ± SD (n = 3) of the percentage of biomass in Aii20J-treated biofilms compared to controls (OD_{590nm}), in which control biofilms are represented by the value "0". Negative values on the y-axis indicate that the Aii20J-treated biofilm was reduced respect to the control, whereas positive values indicate that the Aii20J-treated biofilm was increased respect to the control. Samples used to inoculate the biofilms were obtained from healthy donors (light blue bars) and patients with periodontal disease (PD; dark blue bars). All biofilms were grown for 24h. Subgingival samples were grown in BHI and aerobiosis (**A**) and SCH-K1 and anaerobiosis (**B**). Saliva samples were grown in BHI and aerobiosis (**C**) and SCH-K1 and anaerobiosis (**D**). Asterisks (*) indicate statistical significance (t-tests, *$\alpha = 0.05$). Diamonds (♦) represent

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macroscopical differences between untreated and Aii20J-treated biofilms. In samples P5 (**B**) and H4, H5, H6 and P4 (**D**), marked with an "X" on the x-axis, biofilms could not be quantified. Inserted in each graph is a boxplot (median and interquartile range -IQR-, whiskers range from minimum to maximum) comparing absolute values of biofilm mass (OD_{590nm}) in control and Aii20J-treated samples from healthy donors (n = 7) and PD patients (n = 10). Asterisks (*) indicate statistical significance of the differences between biofilms that were reduced and those that were increased in the presence of Aii20J (Wilcoxon tests, *α = 0.05). AE: aerobiosis. ANE: anaerobiosis. BHI: Brain Heart Infusion. SCH-K1: Schaedler medium supplemented with 0.1 mg L^{-1} of vitamin K1.

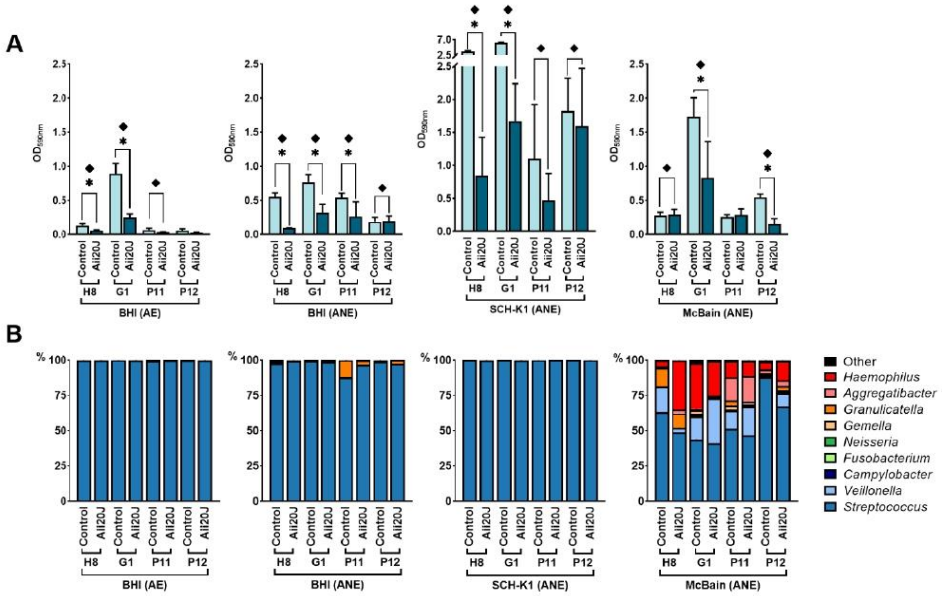


Figure 2. Effect of culture media and conditions on *in vitro* subgingival-derived biofilm formation (A) and bacterial diversity (B) in the presence of Aii20J. Subgingival samples from four patients were grown in the AAA model for 24 h in four different culture conditions: BHI in aerobiosis, and BHI, SCH-K1, and McBain medium in anaerobiosis. (**A**) Biofilm formation, represented as mean ± SD (n = 3) of control and Aii20J-treated biofilms absorbance values (OD_{590nm}). Asterisks (*) indicate statistical significance (t-tests, *α = 0.05). Samples marked with a diamond (♦) presented macroscopical differences between untreated and Aii20J-treated biofilms. (**B**) Relative abundance (in percentage) of the most prevalent genera in the biofilms, as assessed by 16S rRNA gene sequencing. Genera detected in lower relative abundance appear under the category "Other". AE: aerobiosis. ANE: anaerobiosis. BHI: Brain Heart Infusion. SCH-K1: Schaedler medium supplemented with 0.1 mg L^{-1} of vitamin K1.

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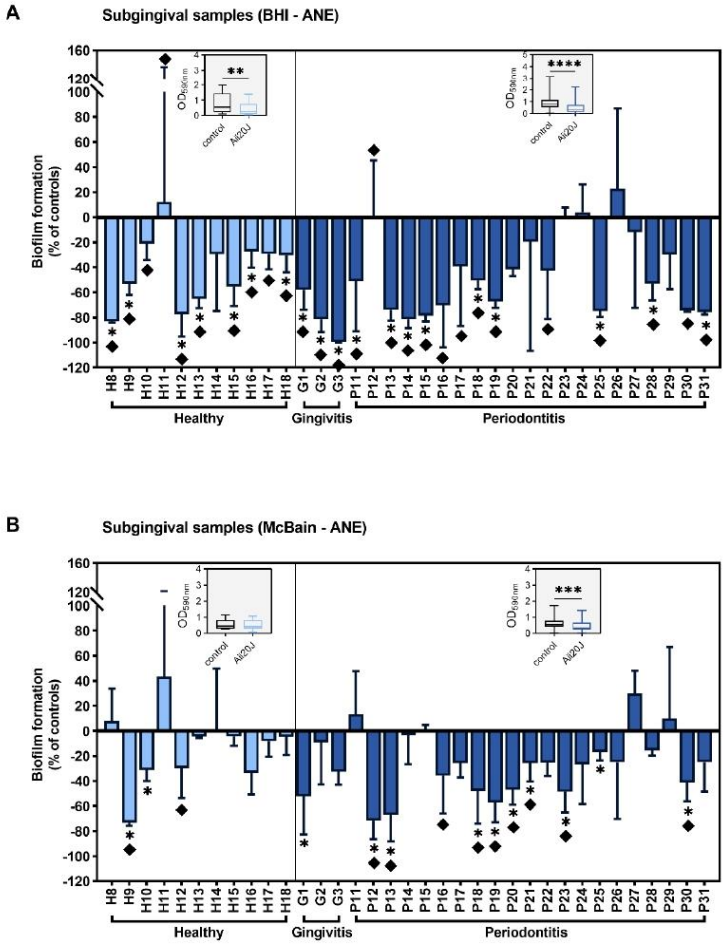


Figure 3. Inhibition of subgingival-derived biofilm formation in the presence of Aii20J. Results are represented as mean ± SD (n = 3) of the percentage of biomass in Aii20J-treated biofilms compared to controls (OD_{590nm}), in which control biofilms are represented by the value "0". Negative values on the y-axis indicate that the Aii20J-treated biofilm was reduced respect to the control, whereas positive values indicate that the Aii20J-treated biofilm was increased respect to the control. Samples used to inoculate the biofilms were obtained from healthy donors (light blue bars) and patients with gingivitis or periodontitis (dark blue bars). All biofilms were grown on the AAA model for 24h. Subgingival samples were cultured in anaerobiosis in BHI (**A**) and McBain medium (**B**). Asterisks (*) indicate statistical significance (t-tests, *α = 0.05). Diamonds (♦) represent macroscopical differences between untreated and Aii20J-treated biofilms. Inserted in each graph is a

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boxplot (median and interquartile range -IQR-, whiskers range from minimum to maximum) comparing absolute values of biofilm mass (OD_{590nm}) in control and Aii20J-treated biofilms from healthy donors (n = 11) and patients with gingivitis and periodontitis (n = 24). Asterisks (*) indicate statistical significance of the differences between biofilms that were reduced and those that were increased in the presence of Aii20J (Wilcoxon tests, **α = 0.01, ***α = 0.001, ****α = 0.0001). ANE: anaerobiosis. BHI: Brain Heart Infusion.

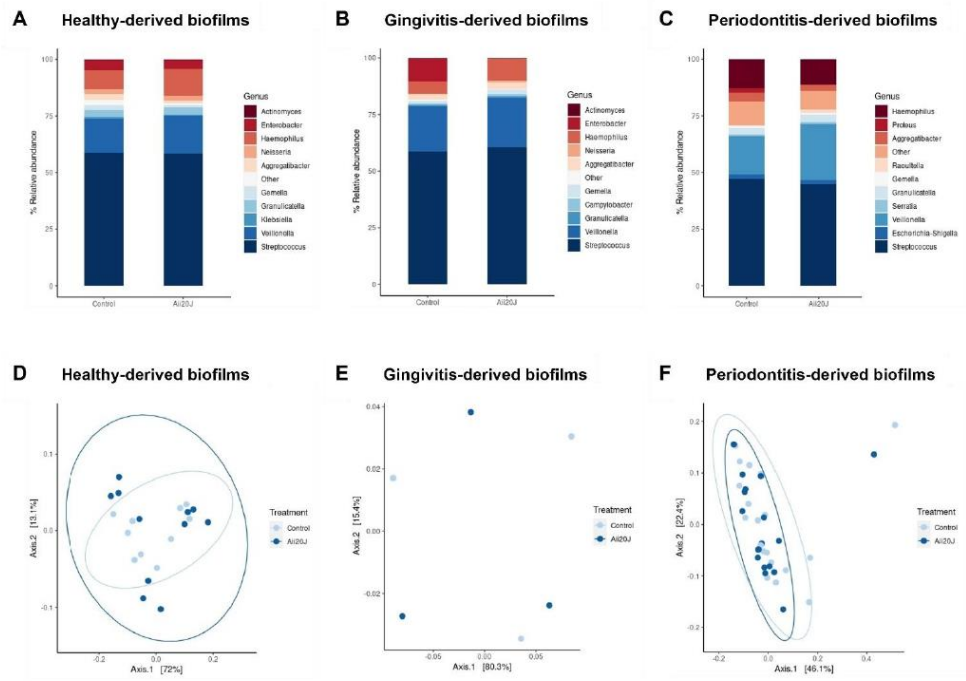


Figure 4. Relative abundance of the bacterial genera identified in the biofilms in the presence of Aii20J. Relative abundance is represented as the percentage of the ten most relatively abundant bacterial genera identified in each biofilm. Genera detected in lower relative abundance appear under the category "Other". Subgingival samples were grown in the AAA model for 24 h in McBain, with and without Aii20J. Samples were obtained from healthy donors (**A**, n = 11), patients with gingivitis (**B**, n = 3), and patients with periodontitis (**C**, n = 19). Principal Coordinate Analysis (PCoA) of weighted Unifrac plots of the microbiome structure of the samples obtained from healthy donors (**D**, n = 11), patients with gingivitis (**E**, n = 3), and patients with periodontitis (**F**, n = 19) are shown.

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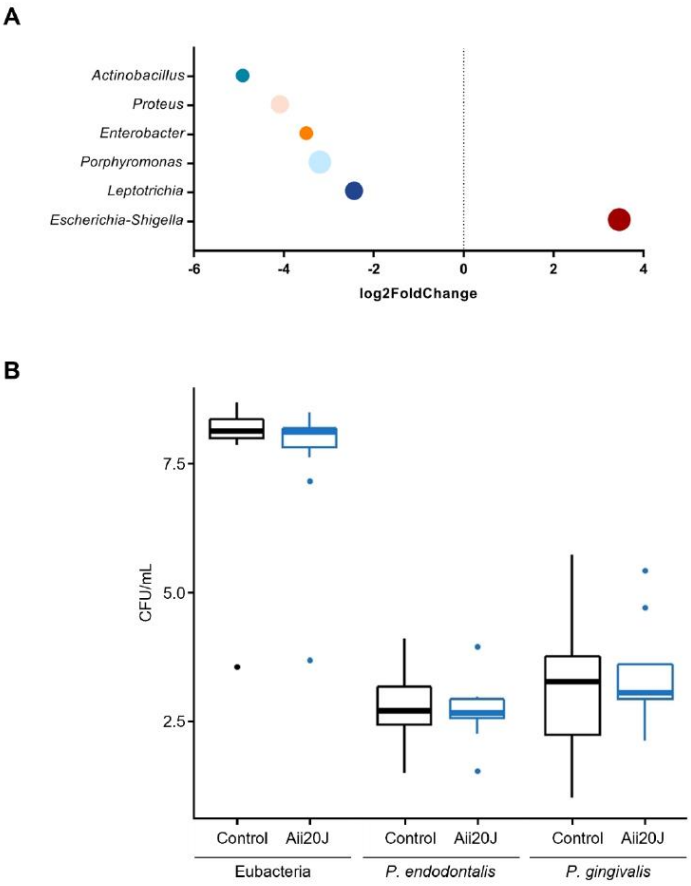
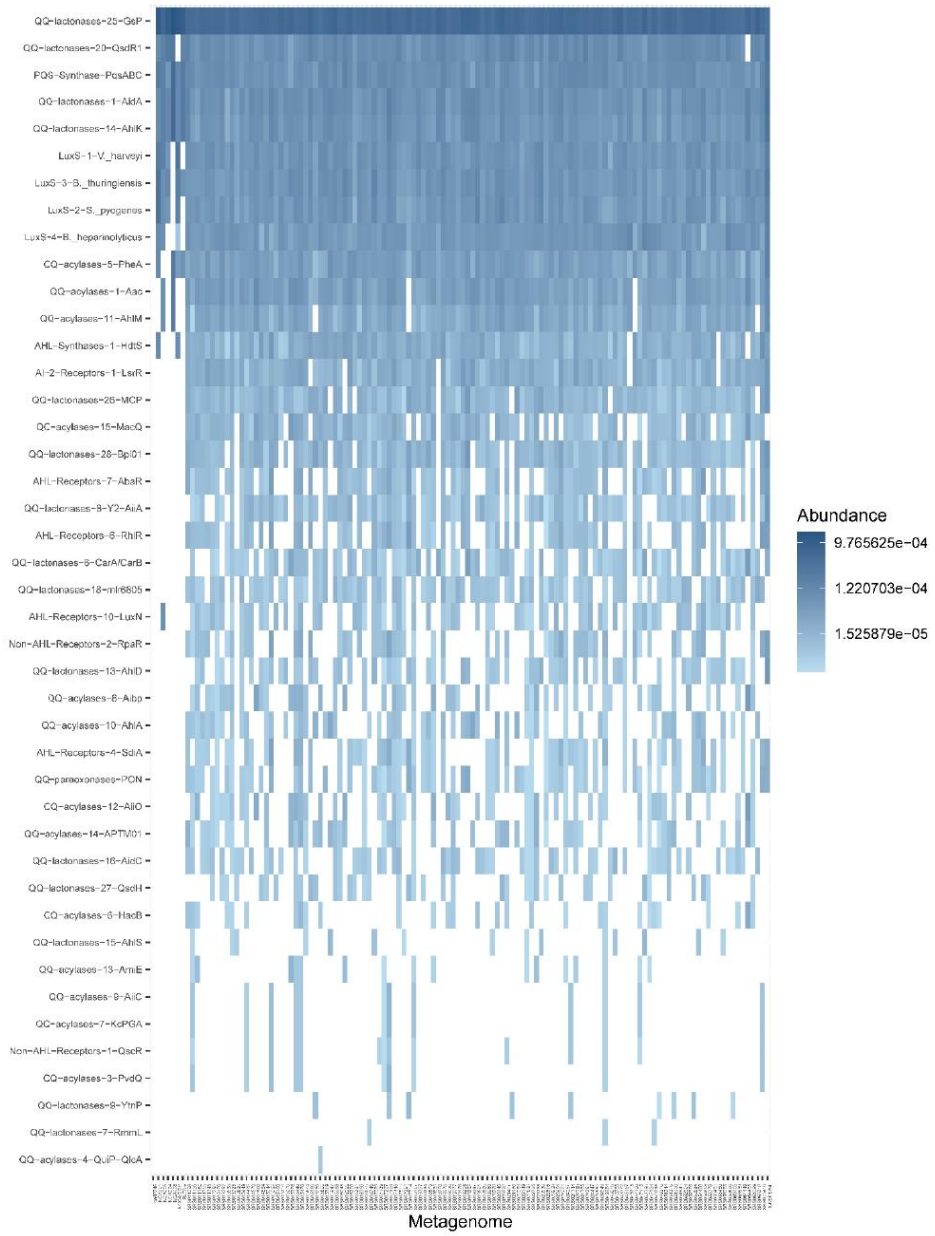


Figure 5. Differential microbial abundance of important genera identified in subgingival, periodontitis-derived biofilms (*n* = 19) in the presence of Aii20J. Samples were grown in the AAA model for 24 h in McBain, with and without Aii20J. **A.** Genera with significant (α = 0.05) differential abundance in Aii20J-treated biofilms are shown. Data were analyzed using the DESeq2 package (Love et al., 2014). Genera with log2 fold change values < 2 or > 2 were considered to be significantly reduced or increased in Aii20J-treated biofilms, respectively. **B.** Quantification, using qPCR, of total 16S rRNA, *P. gingivalis* and *P. endodontalis*, comparing control (black boxes) and Aii20J-treated biofilms (blue boxes). Boxplots represent the median and interquartile range (IQR), and whiskers range from Q1 - 1.5 IQR to Q3 + 1.5 IQR. Mann-Whitney tests (α = 0.05) for independent samples were performed. CFU: colony-forming unit.

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1113 **Figure 6. Abundance of selected quorum sensing and quorum quenching protein families in**
1114 **supragingival shotgun metagenomes.** Results are represented as a heatmap where rows are sorted

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by abundance and columns by non-metric multidimensional scaling distances. The last column on the right is the synthetic subgingival metagenome generated for this study (Supplementary Table SN). The complete set of queried metagenomes is available in Supplementary Table S5. Non-detected proteins are represented by blank spaces. QS: quorum sensing; QQ: quorum quenching; AHL: *N*-acyl-homoserine lactone; AI-2: autoinducer-2.

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The present thesis investigates the potential of inhibiting bacterial cell-cell communication, quorum sensing (QS), to modulate oral biofilm formation. The thesis evaluates the effect of a quorum quenching (QQ) enzyme that degrades N-acylhomoserine lactones, the QS signals of gram-negative bacteria, on in vitro polymicrobial oral biofilms. The potential interactions between AHLs and a gram-positive representative, Enterococcus faecalis, were also investigated. The results presented here support the translation of QQ strategies to in vivo trials for the modulation of oral biofilms. In addition, a more intricate role of AHLs on oral biofilm formation and interactions with gram-positive bacteria is proposed.