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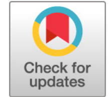

Short-Chain *N*-Acylhomoserine Lactone Quorum-Sensing Molecules Promote Periodontal Pathogens in *In Vitro* Oral Biofilms

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ABSTRACT Acylhomoserine lactones (AHLs), the quorum-sensing (QS) signals produced by a range of Gram-negative bacteria, are involved in biofilm formation in many pathogenic and environmental bacteria. Nevertheless, the current paradigm excludes a role of AHLs in dental plaque formation, while other QS signals, such as AI-2 and autoinducer peptides, have been demonstrated to play an important role in biofilm formation and virulence-related gene expression in oral pathogens. In the present work, we have explored the effect of externally added AHLs on *in vitro* oral biofilm models for commensal, cariogenic, and periodontal dental plaque. While little effect on bacterial growth was observed, some AHLs specifically affected the lactic acid production and protease activity of the biofilms. Most importantly, the analysis of bacterial diversity in the biofilms showed that the addition of C_6-homoserine lactone (C_6-HSL) results in a shift toward a periodontal bacterial composition profile by increasing the relative presence of the orange-complex bacteria *Peptostreptococcus* and *Prevotella*. These results point to a relevant role of AHL-mediated QS in dental plaque formation and might be involved in the development of dysbiosis, the mechanism of which should be further investigated. This finding potentially opens new opportunities for the prevention or treatment of the periodontal disease.

IMPORTANCE Dental plaque is omnipresent in healthy oral cavities and part of our commensal microbial colonization. At the same time, dental plaque is the cause of the most common human diseases, caries and gum disease. Dental plaque consists of billions of microbes attached to the surface of your teeth. Communication among these microbes is pivotal for development of these complex communities yet poorly studied in dental plaque. In the present study, we show that a specific communication molecule induces changes within the community related to the development of gum disease. This finding suggests that interfering with microbial communication may represent an interesting novel strategy to prevent gum disease that should be further investigated.

KEYWORDS dental plaque, quorum sensing, periodontitis, acylhomoserine lactone

Biofilms are sessile microbial communities characterized by cells that are irreversibly attached to a substratum or to each other, embedded in a matrix of self-produced extracellular polymers, and exhibit an altered phenotype with respect to growth rate and gene transcription (1). Dental plaque is a complex biofilm of oral microorganisms, mainly bacteria and fungi, attached to the tooth surface. In most cases, oral biofilms are composed of commensal bacteria, creating a harmless or even beneficial microbial

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community, coexisting with the host in symbiosis. However, in some cases, a shift in ecology and associated functions can result in dysbiosis, eventually leading to a harmful biofilm (2) that is the origin of the development of major human oral diseases, such as caries or periodontitis (3, 4). The complexity of the bacterial community that constitutes the dental plaque is exemplified by the high taxonomic diversity that can be found: between 500 and 1,000 bacterial species have been identified as the core microbiome of the oral cavity, although many of them still remain uncultured (5, 6). Most oral bacteria belong to the bacterial phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria*. Archaea, protozoa, viruses, and fungi are also present in the oral cavity of a healthy adult (7).

Plaque formation is a highly dynamic process of ecological succession, regularly reinitiated by daily oral hygiene measures. Early colonizers adhere to the salivary pellicle, where they influence the environmental conditions, thereby enabling secondary and late colonizers to grow and become part of the biofilm (8). Different types of genes with a wide variety of functions are required for the successful establishment and development of dental plaque. In addition to metabolic and physical interactions, a cell-to-cell communication mechanism known as quorum sensing (QS) is involved. Previous studies have reported the production of QS molecules, such as autoinducer peptides (AIP) and AI-2 signals in monospecific cultures of different oral pathogens (9 to 14). Both Gram-negative and Gram-positive bacteria can synthetize and sense AI-2, known as the "universal" QS signal, which may therefore play a decisive role in multispecies oral biofilm. *luxS*, the gene responsible for the production of AI-2, is conserved among many species of bacteria, including *Aggregatibacter actinomycetemcomitans*, *Streptococcus mutans*, *Streptococcus gordonii*, *Streptococcus oralis*, *Porphyromonas gingivalis*, and other oral pathogens (9–12, 14). Several reports point to an important role of this QS signal in dental plaque formation. AI-2 is essential for mutualistic biofilm growth on saliva as the sole source of nutrients in cocultures of *S. oralis* and *Actinomyces naeslundi* (14), neither of which grows well in monoculture. In addition, *S. gordonii* required the presence of AI-2 to form mixed-species biofilm with the periodontal pathogen *P. gingivalis* (12). The external addition of partially purified AI-2 from *Fusobacterium nucleatum* affected biofilm formation in monospecific and multispecies cultures of *P. gingivalis*, *Treponema denticola,* and *Tannerella forsythia*. In the presence of AI-2, biofilms showed higher biomass, greater average depth, and enhanced coaggregation between bacteria (15). In the case of Gram-positive oral pathogens, AIPs, such as competence-stimulating peptide (CSP), have been identified in various oral streptococci, including *S. mutans*, *S. gordonii,* and *S. intermedius*. CSP is involved in biofilm formation, bacteriocin synthesis, stress resistance, and autolysis (13, 16–18).

N-Acylhomoserine lactones (AHLs) are well-studied QS signals produced by Gram-negative bacteria, although the production of AHLs by the Gram-positive bacterium *Exiguobacterium* strain MPO was described recently (19). AHLs contain a homoserine lactone ring (HSL) linked by an amide bond to a fatty acid (between 4 and 18 carbons). Despite the widespread ability among Gram-negatives to produce AHLs, attempts to establish production of these QS signals by oral bacteria using different bacterial reporter strains have remained unsuccessful (10, 20, 21). AHL-producing bacteria have been isolated from the oral cavity (22–26), although these isolates are not considered members of the core oral microbiome. Additionally, AHLs seem to influence growth and protein expression in *P. gingivalis* (27, 28). However, despite these reports, it is still generally accepted that AHL-based signaling does not play a major role in dental plaque formation during health and disease.

Due to the key role of QS processes for the expression of pathogenic traits, including biofilm formation, the inhibition of QS, a process general known as quorum quenching (QQ), has been proposed as an alternative approach for antimicrobial therapy and for controlling pathogenic bacterial behavior to treat or prevent infectious diseases (29). QQ strategies do not directly interfere with bacterial growth ability, and hence, the probabilities of inducing resistance or tolerance against these mechanisms are lower

TABLE 1 Quantification of AHLs present in two saliva samples obtained from a healthy donor at a 4-month interval

Saliva sample	AHL	AHL concn (ng/ml)
1	OC_8-HSL	1.35
	C_8-HSL	197.68
	HC_{10}-HSL	4.17
2	OC_8-HSL	10.71

(30, 31). Previous studies have reported the successful use of QS inhibitors to control bacterial biofilms (32–37). Since most QS inhibitors described so far act on AHL-mediated QS circuits, the confirmation of a possible role of AHL-type QS signals in dental plaque formation would open new perspectives in the prevention and treatment of oral diseases.

The aim of the present study was to explore a possible role for AHL-based signaling in dental plaque formation by evaluating the effect of externally added AHL on oral biofilms grown *in vitro*. The effect on pathology-related phenotypes in three different, clinically relevant, *in vitro* oral biofilm models for commensal, cariogenic, and periodontal biofilms developed recently (38) was assessed. Lactate production capacity and proteolytic activity were determined as indicators of shifts from commensal toward cariogenic biofilms or periodontal biofilms, respectively (38). In addition, for a selection of AHLs, the effect on microbial composition of commensal and periodontal biofilms was determined using 16S rRNA gene sequencing.

RESULTS

Presence of AHL-type quorum sensing molecules in saliva samples. The saliva of a healthy donor was analyzed for the presence of AHLs by high-performance liquid chromatography–mass spectrometry (HPLC-MS). Two saliva samples were taken at a 4-month interval. Using high-sensitivity HPLC-MS techniques, three different AHLs could be unequivocally identified (see Fig.S1 to S3 in the supplemental material) in the first sample, while only one of them was present in the second sample (Table 1). On both occasions, OC_8-HSL was identified in the samples at concentrations of 1.35 and 10.71 ng/ml of saliva, respectively (Table 1). C_8-HSL and HC_{10}-HSL were identified only in the first sample, indicating variability in the pattern of AHLs present in the saliva with time.

Effect of exogenous AHLs on oral biofilm formation. Very small changes were observed in the number of CFU when the different unsubstituted and oxo-substituted AHLs were added to the culture medium in the three oral biofilm models tested, since in no case was the difference observed with the respective control biofilm higher than 1 order of magnitude (Fig. S4). Still, among the 19 AHLs tested, the exogenous addition of the long-chain AHLs C_{16}-HSL, OC_{16}-HSL, and OC_{18}-HSL significantly reduced ($P < 0.05$) the formation of cariogenic biofilms after 48 h of growth (2.6×10^8 CFU, 2.4×10^8 CFU, and 2.5×10^8 CFU, respectively, versus 4.3×10^8 CFU for the control biofilms) (Fig. S4A). When a commensal biofilm was induced, the signal OC_6-HSL (9.0×10^7 CFU) slightly but significantly ($P < 0.05$) reduced the number of CFU in comparison to that of the control (1.2×10^8 CFU) (Fig. S4B). Lower CFU values were also observed in commensal biofilms when C_{10}-HSLs and C_{12}-HSLs were added, but the difference was not statistically significant in comparison to the respective controls. Periodontal biofilms were the most sensitive to the exogenous addition of AHLs, since both higher and lower numbers of CFU were observed in comparison with the control. In this sense, the short-chain signals C_4-HSL, C_6-HSL, and OC_6-HSL and the very long-chain OC_{18}-HSL significantly promoted biofilm formation (6.8×10^7 CFU, 1.7×10^8 CFU, 9.6×10^7 CFU, and 1.0×10^8 CFU, respectively, versus 5.3×10^7 CFU for the control biofilms) (Fig. S4C). In contrast, C_{14}-HSL clearly decreased the number of CFU (2.6×10^7 CFU versus 7.0×10^7 CFU for the control biofilms) (Fig. S4C).

Effect of exogenous AHLs on lactate production. As expected, the cariogenic control biofilms showed the highest levels of lactate production (4.2 to 9.4 mM) in

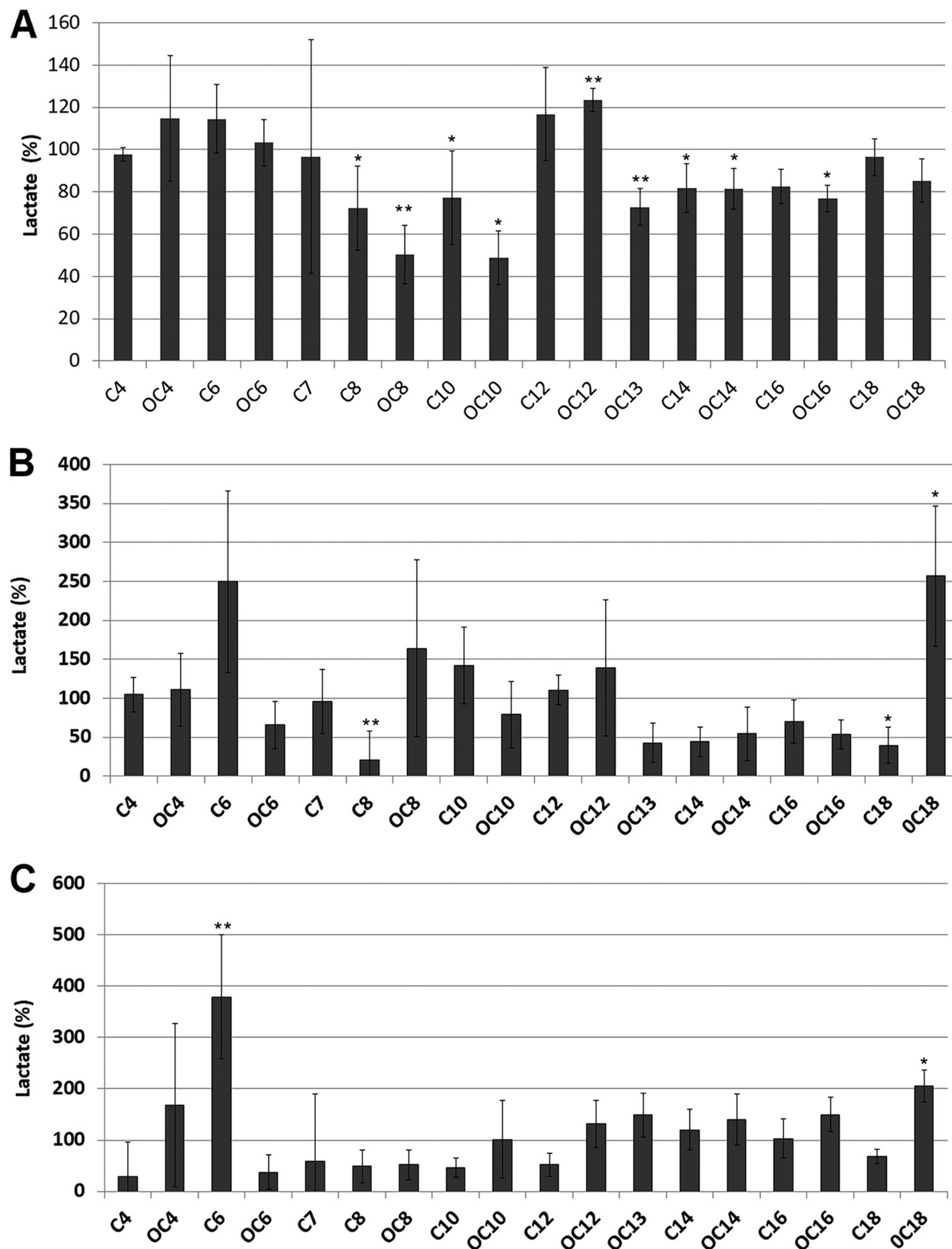

FIG 1 Effect of the AHLs (1 μM) on lactate accumulation (percent in comparison to control cultures) in cariogenic (A), commensal (B), and periodontal (C) biofilms. The increase observed when the signal OC_{18}-HSL is added to commensal and periodontal model biofilms was confirmed in a second independent experiment. Data are presented as means ± standard deviations (SD) (*n* = 4). A significant difference in comparison to untreated controls is indicated as follows: *, Welch's test, $P < 0.05$; **, Welch´s test, $P < 0.01$.

comparison to those of commensal control (0.2 to 2.4 mM) and periodontal control (0.2 to 2.0 mM) biofilms. Significant effects of the addition of AHLs were observed on lactic acid production in the different oral biofilm models when each treated condition was compared to its respective untreated control. The addition of the medium- and long-chain AHLs C_8-HSL, OC_8-HSL, C_{10}-HSL, OC_{10}-HSL, OC_{13}-HSL, C_{14}-HSL, and OC_{14}-HSL caused a reduction in the production of lactic acid (72.3%, 50.3%, 77.2%, 48.8%, 72.8%, 81.9%, and 81.2%, respectively) in comparison to the control (100%) in cariogenic biofilms. Interestingly, the molecule OC_{12}-HSL caused an increase (123.5%) in the production of lactate (Fig. 1A). Shorter AHLs had no significant effect on lactic acid accumulation in cariogenic biofilms. These effects were not observed in the experi-

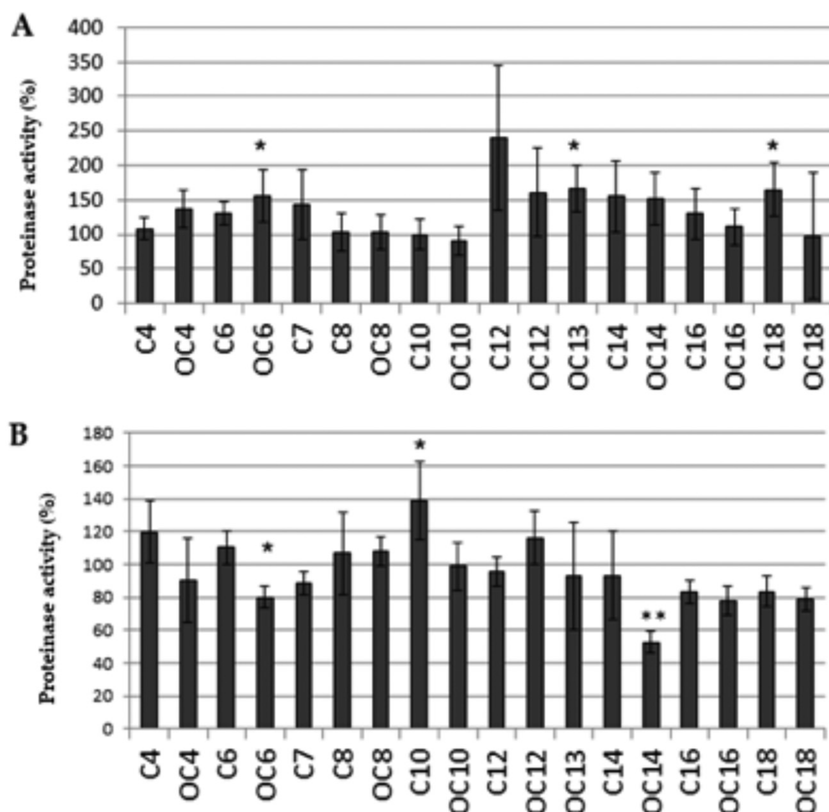

FIG 2 Effect of the addition of AHLs (1 μM) on protease activity (%) in commensal (A) and periodontal (B) biofilms. Data are presented as means $\pm$ SD ($n = 4$). A significant difference in comparisons to untreated controls is indicated as follows: *, Welch´s test, $P < 0.05$; **, Welch´s test, $P < 0.01$.

ments in which lactic acid production in the control cariogenic biofilms was lower than 7.0 mM (data not shown), indicating that robust fermentative activity is needed in the biofilm in order to observe the effect of the addition of AHLs. In the case of commensal biofilms, the addition of C_8-HSL caused a significant decrease in the production of lactate (20.5%) (Fig. 1B). An inhibitory effect on lactic acid production was also observed in commensal biofilms when the long-chain signals between OC_{13}-HSL and C_{18}-HSL were added to the culture medium, although this effect was statistically significant only for the latter. The opposite trend seems to be present in the periodontal biofilm, where, in general, the long-chain AHLs increased lactate accumulation while the short-chain ones decreased lactic acid production (Fig. 1C). The addition of OC_{18}-HSL caused a large increase (256.9%) in lactic acid production in commensal biofilms (Fig. 1B), and the effect was also observed in periodontal biofilms (205.5%) (Fig. 1C). The signal C_6-HSL also produced an increase in lactate accumulation in both commensal and periodontal biofilms. Due to the marginal effects of AHLs on cariogenic biofilms, they were not included in the subsequent sequencing analysis.

Effect of exogenous AHLs on protease activity. Protease activity was measured only in the commensal and periodontal biofilm models. As expected, the protease activity was higher in periodontal control biofilms (2.3×10^7 to 3.4×10^7 relative fluorescence units [RFU]/min) than in commensal control biofilms (7×10^6 to 2.9×10^7 RFU/min). The addition of several short- and long-chain AHLs caused an increase in protease activity of the commensal biofilm in comparison with that of the respective untreated controls, but this increase was statistically significant only for OC_6-HSL, OC_{13}-HSL, and C_{18}-HSL (156%, 166.4%, and 165.1% versus 100% for control biofilms) (Fig. 2A). In contrast, the protease activity was reduced by OC_6-HSL and OC_{14}-HSL in the periodontal biofilm (80% and 52.6%) and increased by C_{10}-HSL (138.8%) (Fig. 2B).

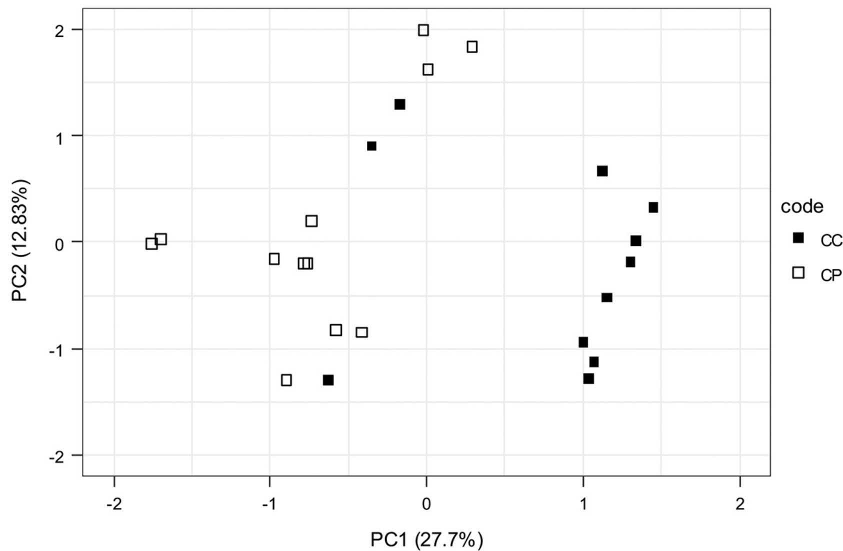

FIG 3 Comparison of species compositions between *in vitro* commensal (control commensal [CC]) and periodontal (control periodontal [CP]) biofilms from 3 independent experiments performed over a period of 3 weeks. Principal-component analysis of commensal biofilms (filled squares) and periodontal biofilms (open squares) grown without exogenously added AHLs was performed. PC1, explaining 27.7% of the variance, separated the commensal and periodontal biofilms (PERMANOVA, F = 6.08, P = 1e−04).

Effect of exogenous AHLs on microbial composition. In order to explain the effects observed in the phenotypic assays, the bacterial composition of the biofilms was determined using 16S rRNA gene sequencing (Fig. S5). The control, commensal, and periodontal biofilms differed significantly in composition (permutational multivariate analysis of variance [PERMANOVA], F = 6.08, P = 1e−04) (Fig. 3). The Shannon diversity for the periodontal biofilms was higher than that for commensal biofilms (commensal biofilm mean of 2.21 versus a periodontal biofilm mean of 2.38; P = 0.03). PC1, explaining 27.7% of the variance, separated the commensal and periodontal biofilms and is represented most notably by a differential abundance of OTU5 and OTU69 (both *Fusobacterium* species) and OTU17 and OTU18 (*Prevotella* and *Alloprevotella*, respectively).

The effects of C_6-HSL, OC_6-HSL, C_8-HSL, OC_8-HSL, C_{18}-HSL, and OC_{18}-HSL on the bacterial composition of commensal and periodontal biofilms were determined and compared with those of their respective intraexperimental untreated control biofilms. When 1 μM AHL was added exogenously during growth of commensal biofilms, the community compositions did not shift, in comparison to their respective controls (Fig. 4A). In contrast, in the periodontal biofilms, a clear shift was observed upon addition of C_6-HSL along the PC1 axis, explaining 31.37% of the variance (Fig. 4B).

When the effect of C_6-HSL was specifically compared to that of the closely related OC_6-HSL, a clear and specific C_6-derived shift in community composition was observed (Fig. 5). The difference was not significant for the commensal biofilms (data not shown); however, it was significant for the periodontal biofilms (PERMANOVA; F = 9.31, P = 2e−04). A clear shift along PC1 (explaining 50.1% variance) can be observed. PC1 is represented most notably by a *Peptostreptococcus* species (OTU9), an *Alloprevotella* species (OTU10), and a *Prevotella* species (OTU23), indicating that these species were largely responsible for the observed shift. Of importance, these species also differentiate commensal biofilms from biofilms grown in a periodontal model (Fig. S6).

DISCUSSION

Cell-to-cell communication is known to play an essential role in microbial pathogenesis, coordinating the physiological behavior, biofilm development, and virulence of several oral pathogens (39). In view of the complexity of the oral microbiota, the

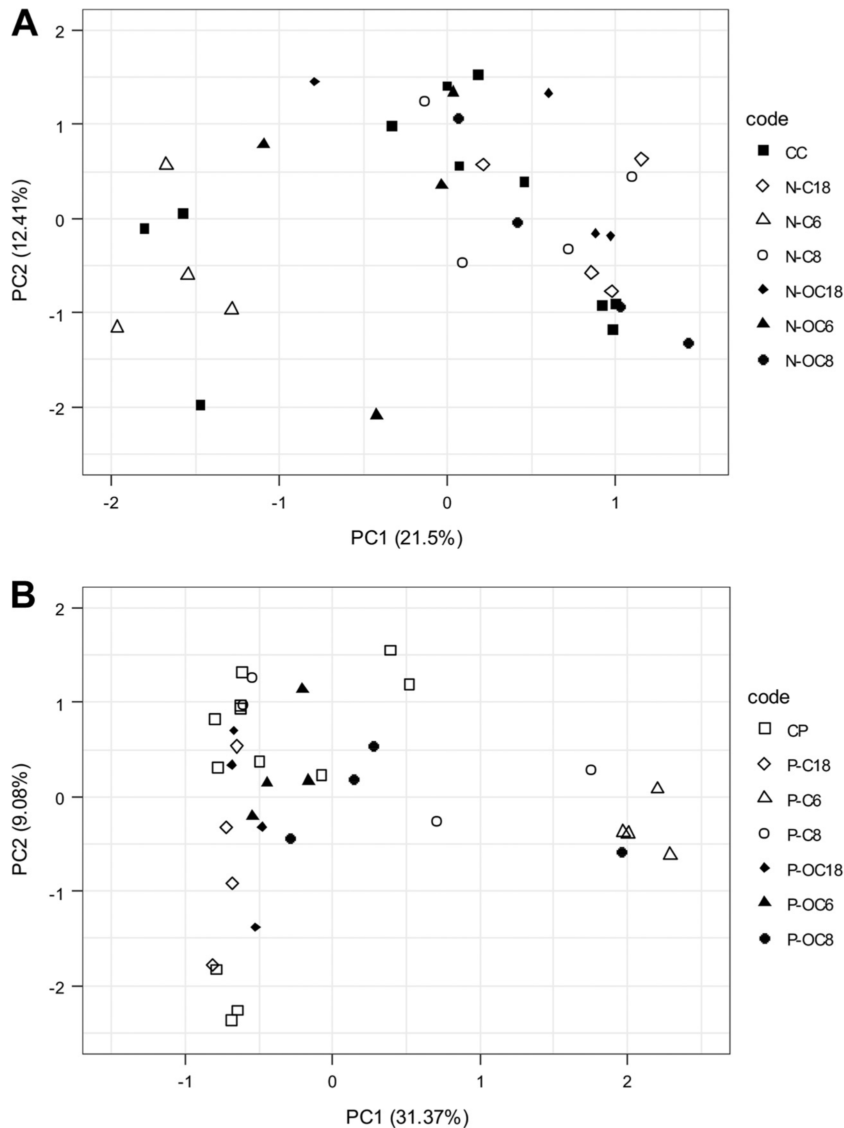

FIG 4 Effect of 1 μM exogenously added AHL on species composition of grown commensal (A) and periodontal (B) biofilms. Filled and open squares represent the untreated controls in commensal and periodontal biofilms, respectively. Open symbols represent the C variant of the HSL, while filled symbols represent the OC variant of the HSL. Triangles, C_6-HSL and OC_6-HSL; circles, C_8-HSL and OC_8-HSL; diamonds, C_{18}-HSL and OC_{18}-HSL in both panels.

characterization of these communication processes is crucial for understanding how oral bacteria interact with each other and for predicting their potential impact on the development of dental plaque-associated oral diseases. Moreover, since the structure and physiological attributes of biofilms confer an inherent resistance to antimicrobial agents (1), the description of the key factors contributing to the formation of pathogenic oral biofilm may allow the identification of novel targets for the development of new antipathogenic strategies. Current intervention strategies attempt to prevent the initial microbial attachment or penetrate the biofilm matrix and kill the associated cells. However, the future approaches to control bacterial biofilms will probably be based on the inhibition of genes involved in biofilm formation (1), in some cases through the disruption of the QS systems (36, 37). Since most QS inhibitors described so far act on AHL-mediated QS circuits, the confirmation of a possible role of AHL-type QS signals in dental plaque formation would open new perspectives in the prevention and treatment of oral diseases.

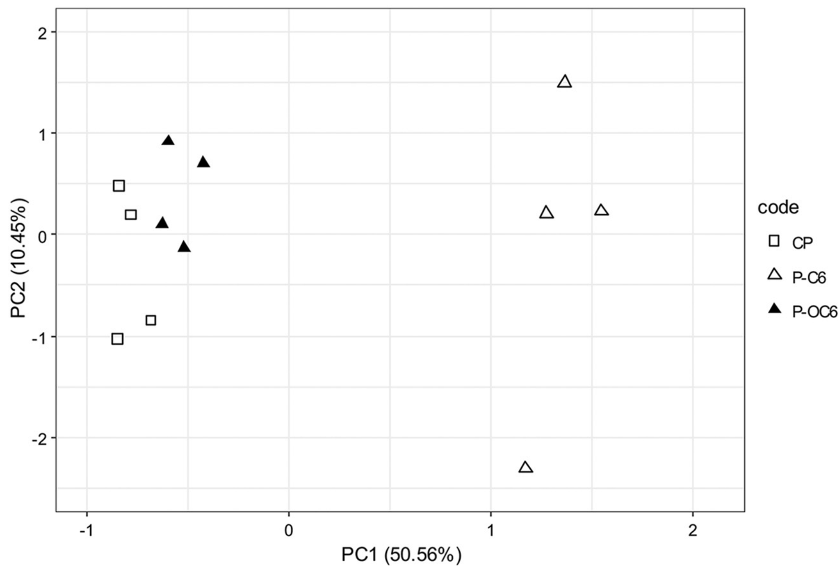

FIG 5 Comparison of the effects of C_6-HSL and OC_6-HSL on species composition of periodontal biofilms. A clear shift along PC1, explaining 50.6% variance in periodontal biofilms, can be observed. The three groups differed significantly (PERMANOVA; $F = 9.31$, $P = 2e{-}04$).

Despite the fact that the current paradigm excludes a role of the AHL-type QS signals in oral biofilm (39, 40) due to the unsuccessful identification of these molecules in pure cultures of oral pathogenic bacteria, important changes mainly in acid lactic production and protease activity were observed when these QS signals were added. These changes do not seem to be derived from gross quantitative changes in biofilm formation, since the observed differences in CFU counts, smaller than 1 log, should not result in a clinically different outcome in the oral cavity (41). Therefore, changes in biofilm formation and metabolic activity observed in the presence of particular exogenous AHLs do not seem to be caused by growth inhibition of bacteria. The potential antimicrobial activity of each AHL at 1 mg/ml on bacterial growth of *S. mutans* was analyzed, but no inhibitory effect was observed (data not shown), supporting the hypothesis that the changes generated in biofilm activity and microbial composition are not derived from growth inhibition. Toxicity of OC_{12}-HSL and its tetramic acid degradation product has been reported for Gram-positive bacteria (42) at concentrations around 10-fold higher than those used in this study. Accordingly, no inhibitory activity of oxo-substituted AHL derivatives was observed in the oral biofilm models (see Fig. S4 in the supplemental material).

While only small changes could be observed in CFU numbers, important changes in metabolic activities were found after the addition of specific AHLs in the three oral biofilm models. These phenotypic changes without a concurrent large effect on bacterial growth are typical of a QS-derived gene regulation process. All together, these data strongly support the idea of a potential role of the AHL-type QS molecules in the oral cavity. The wide range of responses to the addition of the signals, with biofilm models responding in opposite directions to the addition of very similar AHL signals, may be derived from the specificity of some of the QS receptors. It is well known that while the cognate AHLs can activate the QS circuits, noncognate AHLs, even of a very similar structure, may act to inhibit the same receptor (43, 44). The signaling molecules C_6-HSL, C_8-HSL, C_{10}-HSL, and C_{16}-HSL, which generated the most important changes in the model biofilms, have been previously described in bacteria isolated from the oral environment (23–26). In contrast, other signals such as OC_6-HSL, C_{18}-HSL, and OC_{18}-HSL, which showed the highest effect on the studied phenotypes, have not been described in the oral environment so far. Moreover, C_{14}-HSL, one of the AHL signals that generated changes in the model oral biofilms, has been previously reported for causing

changes in growth and protein expression in *P. gingivalis* (27). In that case, the concentration used was 2 orders of magnitude higher than the one used in the present work, 1 μM, which is in range with the concentration of QS molecules produced by pathogens (45) and has previously shown effects on the non-AHL-producing foodborne pathogens *Escherichia coli* and *Salmonella enterica* (46). Regarding the production of AHLs by oral pathogens, previous studies have been unable to obtain evidence of the production of AHLs by *P. gingivalis* and other oral pathogens by using the supernatants of monospecific cultures (10, 21). Beyond the limitations of the use of biosensors for the detection of AHLs, it should be taken into account that production of AHLs may change depending on culture medium and conditions (47, 48) and also depends on other factors, such as surface attachment or cell-to-cell adherence, which activates AHL synthesis. For example, the gene A1S_0114 of *Acinetobacter baumannii*, encoding a small acidic acyl carrier protein (ACP) essential for AHL synthesis, is expressed at high levels in biofilms and downregulated in planktonic cells (49), further linking cell adherence and QS expression. Moreover, AHLs can be detected only in the culture medium of this pathogen under static conditions (48). This could explain that AHLs have not been found so far in pure cultures of oral pathogens.

We cannot discard that AHL producers and sensors may play a key role in biofilm formation despite representing a minority in the biofilm community. Some bacteria have LuxR homologs, called LuxR orphans, which do not have an associated LuxI autoinducer synthase but can interact with the autoinducers synthetized by other bacteria (47, 50). As an example, although *E. coli* does not produce AHLs, it possesses a LuxR homolog, called SdiA, and uses various AHLs as switches for its regulatory activity (51). Also, although the AHL-type QS molecules are considered typical of Gram-negative bacteria, previous reports indicate the ability of Gram-positive bacteria not only to produce them (19) but also to respond to them (52, 53). In the genome of *Streptococcus mutans*, the predicted product of the *smu.46* gene showed features of the LuxR family of regulatory proteins, suggesting the existence of a LuxR orphan (53). Indeed, a previous study with *Staphylococcus aureus* has demonstrated the interaction of this bacterium with the OC_{12}-HSL produced by *Pseudomonas aeruginosa* in a saturable and specific manner, inhibiting the production of exotoxins and enhancing protein A expression (52). Our results strongly indicate the existence of complex QS networks in the oral biofilm beyond the accepted model in which the oral bacterial communication is mediated by AIPs and AI-2 (39, 40).

The 16S rRNA gene sequencing data indicate that exogenous C_6-HSL has the ability to change the microbial composition of *in vitro* oral biofilms. Interestingly, OC_6-HSL does not induce a similar shift in composition. This specificity of C_6-HSL points toward the involvement of a specific C_6-HSL binding protein (possibly a receptor) that detects the presence of this signal. Surprisingly, the effect of the addition of C_6-HSL on the microbial composition of both commensal and periodontal biofilms was accompanied by an increase in lactate production in both models (Fig. 1). In contrast, no significant effect on protease activity was observed (Fig. 2), indicating that this phenotype is not sensitive enough to allow the detection of important shifts in the population. C_6-HSL increases the abundance of *Alloprevotella*, *Peptostreptococcus*, and *Prevotella* species in periodontal biofilms, the latter two being important members of the orange complex as defined by Socransky et al. (54). This could be a direct effect, for instance, by increasing the biofilm-forming capacity of these species. Alternatively, the reduction of the fitness of bacterial species that compete with, or have an antagonistic activity toward, *Peptostreptococcus* and *Prevotella* species could also explain this effect. Further research on the mechanism of the observed increased abundance is needed to determine this. If C_6-HSL induces the presence of certain periodontal pathogens directly, inhibition of C_6-HSL sensing could become a viable option to impact the shift from a commensal to a periodontal biofilm. The QQ strategies to prevent the biofilm formation by oral pathogens constitute an attractive alternative, since these approaches do not interfere with bacterial growth, and hence, the probabilities of inducing resistance or tolerance against these mechanisms are lower. In fact, it has

been demonstrated that QQ strategies increase the susceptibility to antibiotics in biofilm-forming pathogens (55–57). Further studies are required to assess the possible role of the AHL-type QS molecules in the virulence of oral pathogens and to explore the viability of the application of these strategies in the field of oral health.

The results indicate a potential role of AHLs in the development of dysbiosis related to periodontal diseases. Further studies are required to confirm the role of the AHL-type QS molecules in dental plaque formation *in vivo*, which would open the possibility of applying QQ strategies to prevent the development of dysbiosis in oral biofilms leading to the development of oral diseases.

MATERIALS AND METHODS

Extraction and identification of AHLs by HPLC-MS. The identification of AHLs was done in different acidified saliva samples from the same healthy donor who provided a written informed consent that was approved by the Comité Ético de Investigación Clínica de Galicia (protocol 2009/319 modified in July 2017). The extraction of the sample (approximately 0.5 ml) was performed twice in an appropriate volume of organic solvents, ethyl acetate, and dichloromethane, and then it was evaporated to dryness at 40°C. AHLs present in the oral samples were reconstituted in acetonitrile and quantified by HPLC-MS methodology (45). Pure AHLs covering the whole range of AHL lengths (from C_4-HSL to C_{18}-HSL), both substituted and unsubstituted, were obtained from Sigma-Aldrich and from the University of Nottingham and used as external standards for quantification.

Test compounds. QS AHL type molecules were used at 1 μM for this study. They included *N*-butanoyl-L-homoserine lactone (C_4-HSL), *N*-oxobutanoyl-L-homoserine lactone (OC_4-HSL), *N*-hexanoyl-L-homoserine lactone (C_6-HSL), *N*-oxohexanoyl-L-homoserine lactone (OC_6-HSL), *N*-heptanoyl-L-homoserine lactone (C_7-HSL), *N*-octanoyl-L-homoserine lactone (C_8-HSL), *N*-oxooctanoyl-L-homoserine lactone (OC_8-HSL), *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), *N*-oxodecanoyl-L-homoserine lactone (OC_{10}-HSL), *N*-dodecanoyl-L-homoserine lactone (C_{12}-HSL), *N*-oxododecanoyl-L-homoserine lactone (OC_{12}-HSL), *N*-oxotridecanoyl-L-homoserine lactone (OC_{13}-HSL), *N*-tetradecanoyl-L-homoserine lactone (C_{14}-HSL), *N*-oxotetradecanoyl-L-homoserine lactone (OC_{14}-HSL), *N*-hexadecanoyl-L-homoserine lactone (C_{16}-HSL), *N*-oxohexadecanoyl-L-homoserine lactone (OC_{16}-HSL), *N*-octadecanoyl-L-homoserine lactone (C_{18}-HSL), and *N*-oxooctadecanoyl-L-homoserine lactone (OC_{18}-HSL). The compounds were obtained from Sigma-Aldrich (St. Louis, MO) and the University of Nottingham.

Biofilm formation. Saliva was collected on ice from a single healthy donor, as previously described (38). The saliva was diluted 2-fold with sterile glycerol, aliquoted, and stored at −80°C.

Biofilms were grown in the Amsterdam active attachment model (AAA model) (58) assembled with round glass coverslips (diameter, 12 mm). The model was differentiated toward commensal, cariogenic, or periodontal biofilms by using one of three culture media (38): (i) for commensal biofilms, buffered semidefined McBain medium (59), containing 2.5 g/liter mucin, 2 g/liter Bacto peptone, 2 g/liter Trypticase peptone, 1 g/liter yeast extract, 0.35 g/liter NaCl, 0.2 g/liter KCl, 0.2 g/liter $CaCl_2$, 1 mg/ml hemin, and 2 mg/liter vitamin K1 with PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] at pH 7.0; (ii) for cariogenic biofilms, buffered semidefined McBain medium with 0.2% sucrose; and (iii) for periodontal biofilms, buffered semidefined McBain medium with 10% fetal calf serum (FCS).

All biofilms were inoculated for 8 h at 37°C under anaerobic conditions using a 1:50 dilution of saliva in the model-specific medium. Cariogenic biofilm was grown in the presence of sucrose for a total of 48 h, with twice daily (at 8 and 16 h) medium refreshments as described previously (38). Commensal and periodontal biofilms were grown for a total of 9 days. After an initial 8-h inoculation, the medium was refreshed every 24 h, except for the weekends.

The respective AHLs were added during all phases of biofilm formation, including the initial 8-h inoculation step.

Acid production assay. Lactic acid production by the biofilms was determined prior to harvesting to estimate the cariogenic phenotype (38, 58). The biofilms on coverslips were placed in a 24-well plate containing 1.5 ml of buffered peptone water (BPW) with 0.2% sucrose in each well. Acid formation was allowed for 3 h at 37°C under anaerobic conditions. The amount of lactic acid produced was analyzed using the colorimetric assay described previously (60).

Protease activity. To quantify the protease activity of the biofilms, the fluorescence resonance energy transfer (FRET) assay was used (61, 62). The probe used in the present study was PEK-054 (63), a probe for total protease activity (kindly provided by F. J. Bikker, Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, The Netherlands). The spent medium was filter sterilized using 0.2-μm-pore-size filters and stored at −20°C until needed. Wells of clear-bottom 96-well plates were filled with 100 μl of Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.6) and 100 μl of sterile spent medium. Four microliters of substrate PEK-054 (800 μM) was added to each well to a final concentration of 16 μM. Sterile fresh culture broth was used as the control. The fluorescence of each well was read for 2 h with 5-min intervals on a fluorescence microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Relative fluorescence (RF) values were obtained after correction against the culture broth control. The protease activity was defined in RFU per minute. The protease activity was measured in commensal and periodontal biofilms.

Harvesting of biofilms. All biofilms were harvested by transferring the glass coverslips into 2 ml of phosphate-buffered saline (PBS), as previously described (38). Biofilms were sonicated to disperse

following the phenotypic assays, to allow CFU determination and subsequent DNA isolation for sequencing analysis.

Biofilm biomass estimation. The numbers of CFU were determined to estimate biofilm formation (38, 58). Serial dilutions in cysteine peptone water (CPW) of the dispersed biofilms were plated on tryptic soy agar blood plates (TSA-b). The plates were incubated anaerobically for 96 h at 37°C, and the CFU were counted. Since not all AHLs could be tested simultaneously for each model, the results are expressed as a percentage with respect to the corresponding control. Average CFU values for control cultures were $6.47 \times 10^7 \pm 2.57 \times 10^7$ for cariogenic, $8.59 \times 10^7 \pm 4 \times 10^7$ for commensal, and $1 \times 10^8 \pm 0.8 \times 10^8$ for periodontal biofilms.

DNA isolation and microbiome analysis. Commensal and periodontal biofilms were used for microbiome analysis. Total DNA was isolated and purified as previously described (38). Bacterial DNA concentration was determined by quantitative PCR (qPCR), using a universal primer-probe set targeting the 16S rRNA gene (64). Next, 1 ng of DNA was used to amplify the V4 hypervariable region of the 16S rRNA gene, as described previously (65) except that 33 amplification cycles were performed. The amplicons were pooled equimolarly and purified from agarose gel (Illustra; GE Healthcare, United Kingdom). Paired-end reads of 251 nt were generated using the Illumina MiSeq platform and Illumina MiSeq reagent kit v3 (Illumina, Inc., San Diego, CA, USA) at the VUmc Cancer Center Amsterdam (Amsterdam, the Netherlands). The sequence data were processed as described previously (66); however, 10% mismatches in the overlap here translates to a maximum of 25 mismatches. Seventy-five samples (including controls), represented by 1.87 million read pairs, were processed; of these, 72.3% passed the merging and quality-filtering steps (maximum expected error, 0.5). Of these quality-filtered reads, 91.0% mapped to the final OTU table.

Sequencing data analysis. The OTU table was randomly subsampled at 11,000 reads/sample and in R v3.5.1 (67) using the microbiome v1.4.0 (68), phyloseq v1.26.0 (69), and vegan v2.5-3 (70) packages from R. The OTU table was $\log_2$ transformed to ordinate the data by principal-component analysis (PCA) into two dimensions. The PCA coordinates were eigenvalue scaled. Plots were generated with ggplot2 v.3.1.0 (71).

Statistical analysis. Due to the large number of conditions obtained, it was not possible to test all of them in a single experiment. Therefore, several batches of experiments, all with their own untreated controls, were performed using the same frozen saliva inoculum. A Welch's t test was performed to determine the statistical significance of the effect of the addition of each AHL on biofilm formation (CFU), lactic acid production, and protease activity between each treated well and its respective untreated control. Significant differences were determined at P values of <0.05 and <0.01.

Permutational multivariate analysis of variance (PERMANOVA) using the Bray-Curtis distance was performed in R on the transformed OTU table using 9,999 permutations. Differences in Shannon diversities were tested in R using the Wilcoxon rank sum (Mann-Whitney) test.

Data availability. The data are available in the NCBI BioProject database under accession number PRJNA573890.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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