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ORIGINAL ARTICLE

A novel compound to maintain a healthy oral plaque ecology *in vitro*

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Objective: Dental caries is caused by prolonged episodes of low pH due to acid production by oral biofilms. Bacteria within such biofilms communicate via quorum sensing (QS). QS regulates several phenotypic biofilm parameters, such as biofilm formation and the production of virulence factors. In this study, we evaluated the effect of several QS modifiers on growth and the cariogenic potential of microcosm oral biofilms.

Methods: Biofilms were inoculated with pooled saliva and cultured in the presence of sucrose for 48 and 96 h. QS modifiers (or carrier controls) were continuously present. Lactic acid accumulation capacities were compared to evaluate the cariogenic potential of the biofilms. Subsequently, biofilm growth was quantified by determining colony forming unit counts (CFUs) and their ecology by 16S rDNA-based microbiome analyses. The minimal inhibitory concentration (MIC) for several *Streptococcus* spp. was determined using microbroth dilution.

Results: Of the tested QS modifiers only 3-oxo-*N*-(2-oxocyclohexyl)dodecanamide (3-Oxo-N) completely abolished lactic acid accumulation by the biofilms without affecting biofilm growth. This compound was selected for further investigation. The active range of 3-Oxo-N was 10–100 μM. The homologous QS molecule, acyl homoserine lactone C12, did not counteract the reduction in lactic acid accumulation, suggesting a mechanism other than QS inhibition. Microbial ecology analyses showed a reduction in the relative abundance of *Streptococcus* spp. in favor of the relative abundance of *Veillonella* spp. in the 3-Oxo-N exposed biofilms. The MIC of 3-Oxo-N for several streptococcal species varied between 8 and 32 μM.

Conclusion: 3-Oxo-N changes the ecological homeostasis of *in vitro* dental plaque. It reduces its cariogenic potential by minimizing lactic acid accumulation. Based on our *in vitro* data, 3-Oxo-N represents a promising compound in maintaining a healthy, non-cariogenic, ecology in *in vivo* dental plaque.

Keywords: In vitro *biofilms*; *caries*; *quorum sensing*; *healthy ecology*; *3-oxo*-N-*(2-oxocyclohexyl)dodecanamide*; *dental plaque*

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Despite decades of intensive research and improved preventive strategies, caries is still the most prevalent dental disease in the world; nearly all adults have experienced dental cavities (1). Caries is caused by bacteria in oral biofilms that convert fermentable dietary carbohydrates to organic acids. This creates an acidic environment in which first the enamel surface of the teeth and subsequently dentine is dissolved. Caries eventually develops upon prolonged and repeated episodes of increased acidity.

Oral biofilms consist of many microbial species embedded in a polymeric matrix. They use intricate mechanisms to manipulate each other's behavior (2). Microbes can communicate with their community members via sophisticated cell-density-dependent communication systems termed 'quorum sensing' (QS) systems (3). QS systems generally consist of a signaling molecule and a specific receptor. Specificity of the molecule and of the receptor varies. Gram-negative bacteria generally use LuxI/LuxR-type QS systems to generate species-specific acylated

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homoserine lactone (HSL) autoinducer. Gram-positive bacteria use species-specific autoinducing peptides (AIPs) to communicate within biofilms. Apart from these species-specific signals, microbes in biofilms can also register the presence of other species. Interspecies signaling, mediated via the autoinducer-2 (AI-2) produced by LuxS, is a widespread interspecies QS signal (3). LuxS has been detected in many Gram-positive as well as Gram-negative oral bacteria. McNab and coworkers (4) showed that *Streptococcus gordonii* is not able to form mixed-species biofilms with *Porphyromonas gingivalis* in the complete absence of AI-2. If only one of the species was LuxS-impaired, mixed biofilms could still form, suggesting that AI-2 of either of the species complemented the LuxS mutation in the other (4). In addition to interspecies signaling, AI-2 also functions in intraspecies signaling. Virulence of *P. gingivalis* depends partly on the ability to stimulate the inflammatory response of the host, and this is significantly reduced in a *P. gingivalis* LuxS mutant (5).

Due to the extracellular nature of QS signals, these signals can also be detected by many other organisms in the direct vicinity, including fungi and even the host. For instance, the fungus *Candida albicans* is able to detect the AIP produced by *Streptococcus mutans* (6) and IA-2 produced by *Aggregatibacter actinomycetemcomitans* (7). HSL-based QS signals are detected by macrophages of the host and modulate the immune response (8). Therefore, modulation through QS signals is capable of influencing the complete breath of ecology.

Thus, QS are ubiquitous, extracellular signals that affect virulence, biofilm formation, and biofilm interactions in single species, dual species, and polymicrobial oral biofilms. While the importance of QS in modulating the pathology of complex oral communities is recognized, this has not yet been evaluated in detail. In this study, we describe the effect of several QS modifiers on biofilm growth and cariogenic potential of microcosm oral biofilms.

Materials and methods

Inoculum collection and strains

Whole human stimulated saliva was collected on ice and from 10 (self-reported) healthy donors who refrained from oral hygiene for 24 h before the donation (9). Saliva was two-fold diluted with 60% sterile glycerol, aliquoted, and stored at −80°C. A pooled sample was prepared before use by mixing 200 l of thawed saliva from each donor and vortexing for 30 s. Pooled saliva was diluted 50-fold for inoculation of the biofilm model.

Reference strains of *Streptococcus* species used for determination of the minimal inhibitory concentration (MIC) were *S. mutans* UA159 (ATCC700610), C180-2, C67-1 and HG723, *S. mitis* LMG14557, *S. gordonii* ATCC35105, *S. salivarius* 57 I, *S. sanguinis* DSM20567, *S. oralis* ATCC35057, and *S. sobrinus* DSM20742.

Test compounds

Different QS modifiers were selected for this study. The molecule 3-oxo-*N*-(2-oxocyclohexyl)dodecanamide (3-Oxo-N) is a structural homologue of the *N*-3-oxo-dodecanoyl-L-homoserine lactone (C12-HSL) and known to inhibit biofilm formation in *Pseudomonas aeruginosa* (10). The (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (Furanone C30) is known to inhibit AI-2 activity in *S. mutans* (11). Brominated furanones also inhibit AI-2 QS signals (12). Therefore, 3,4-dibromo-2(5*H*)-furanone (3,4-dibromo) was selected. As a control, the effect of the AI-2 precursor (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) was evaluated.

All compounds (Table 1), except DPD and C12-HSL, were obtained from Sigma Aldrich (St. Louis, MO). Synthetic DPD and C12-HSL were kindly provided by Dr. Meijler, Ben Gurion University, Israel. The compounds were dissolved in dimethylsulfoxide (DMSO) and stored at −20°C. The compounds, or carrier controls, were continuously present at the indicated concentrations during biofilm growth, but not during further phenotypic analysis (i.e. lactic acid accumulation assay).

Biofilm formation

The well-established Amsterdam Active Attachment Model [AAA-model (13)], assembled with 12 mm glass coverslips (Menzel, Braunschweig, Germany), was used for *in vitro* oral biofilm formation. Buffered semi-defined McBain medium (13), supplemented with 0.2% sucrose, was used for biofilm growth. Four replica biofilms per condition were grown for 48 and 96 h in continuous presence of the test compounds (Table 1), as described previously (13). Briefly, the AAA-model was inoculated

Table 1. Compounds tested in an *in vitro* oral plaque biofilm model

	Compound	Concentration (μM)	Reference
3-Oxo-N	3-Oxo-*N*-(2-oxocyclohexyl)dodecanamide	0.01–100	(10)
3,4-Dibromo	3,4-Dibromo-2(5*H*)-furanone	10	
Furanone C30	(*Z*-)-4-Bromo-5-(bromomethylene)-2(5*H*)-furanone	10	(11)
C12-HSL	*N*-(3-Oxododecanoyl)-L-homoserine lactone	10–100	(10)
DPD	(*S*)-4,5-Dihydroxy-2,3-pentanedione	10	

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with McBain with 0.2% sucrose, test compound or DMSO as carrier control, and pooled saliva, and incubated anaerobically at 37°C for 8 h allowing the microbes to attach to the glass surface. After the 8 h attachment period, the inoculation medium was refreshed by transferring the lid with the coverslips to a new plate, containing fresh, sterile McBain medium with 0.2% sucrose and test compound or DMSO, and *in vitro* oral biofilms were grown for 16 h. This refreshment routine was repeated daily until the day of harvesting.

Biofilm quantification

To harvest the biofilms, the glass coverslips were transferred into 2 ml phosphate buffered saline. Biofilms were dispersed by sonication on ice for 1 min with a pulse rate of 50%, pulses of 1 sec and a vibration amplitude of 40% (Vibracell VCX130, with a maximum of 130 Watts and 20 kHz) (9).Total anaerobic colony forming units (CFUs) were determined to estimate the amount of biofilm formation (13). In brief, dispersed biofilms were serially diluted and plated on tryptic soy agar blood plates. After 96 h of anaerobic incubation at 37°C, CFUs were determined by counting the number of colonies for each dilution.

Acid accumulation assay

Lactic acid accumulation by the biofilms was determined prior to harvesting, to estimate the cariogenic potential of the polymicrobial communities (13). Biofilms were incubated anaerobically (37°C) in sterile buffered peptone water (BPW) with 0.2% sucrose for 3 h. The total amount of lactic acid accumulated in 3 h (in mM) per biofilm was determined using a colorimetric assay (14).

Competition of QS molecule and inhibitor

The effect of the natural QS molecule acyl homoserine lactone C12 (C12-HSL) on the inhibition of lactic acid accumulation by 3-Oxo-N was evaluated using a checkerboard-like approach. Biofilms were grown for 48 h in the presence of 0, 25, or 100 μM 3-Oxo-N and competing concentrations of 0, 25, or 100 μM of C12-HSL. After growth, the cariogenic potential of the biofilms was determined using the acid accumulation assay.

DNA isolation and amplicon sequencing

Two of the four replica biofilms of each condition were used for microbiome analysis. DNA was isolated as described previously, using phenol bead beating followed by Agowa nucleic acid isolation according to the manufacturer's instructions (LGC Genomics, Mag mini kit) (9). Briefly, cells were mixed with TRIS-saturated phenol, pH 8, 0.1 mm zirconium beads, and Mag lysis buffer and were mechanically disrupted four times at 1,200 rpm for 2 min. The DNA containing phase was added to binding buffer and magnetic titanium beads. After washing the beads, according to the Agowa Mag mini DNA extraction protocol, the DNA was eluted from the beads with 63 μl elution buffer (Agowa). The amount of bacterial DNA after purification was determined by QPCR, using a universal primers-probe set targeting the 16S rRNA gene (15).

For amplification of the V4 hypervariable region of the 16S rRNA gene 100 pg DNA was used as described previously (15) except that 5 μM of each primer was used and 30 cycles were performed. Using Fragment analyzer, the generated amplicons were checked for quality, pooled equimolarly, and purified from agarose gel (Qiagen, Roermond, The Netherlands). Paired-end sequencing of the amplicons was conducted on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) using the Illumina MiSeq reagent kit V2 to generate 200-bp paired-end reads.

Data processing and analysis

The sequencing data were processed in mothur (16) as described in supplementary methods (Supplementary File S1). Briefly, after merging the reads, the sequences were aligned, pre-clustered, and chimeric reads were removed. Sequences occurring less than 10 times were removed as well. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the ribosomal database project (RDP), naïve Bayesian classifier (17), and the mothur-formatted version of the RDP training set v.9 (trainset9_032012). A consensus taxonomy was assigned to the OTU, bases on the taxonomy of its member sequences. In addition, the OTU-representative sequences were assigned a species name based on the human oral microbiome database (18). A genus-level summary was used to calculate the percentage abundance of all genera.

Minimal inhibitory concentration determination

The MIC of 3-Oxo-N for different streptococcal species was tested using reference strains in pure culture. One colony of each *Streptococcus* species was cultured overnight in 5 ml brain-heart infusion (BHI) (BD Diagnostic Systems, Sparks, MD) at 37°C. Cultures were diluted and mixed in a 96-well plate 1:1 with a serial dilution of 3-Oxo-N in BHI (final concentration of 1×10^7 CFU/ml *Streptococcus* and 2–1,000 μM 3-Oxo-N). DMSO was used as carrier control. The plate was incubated for 24 h anaerobically at 37°C and optical density was measured at 600 nm. The lowest concentration without significant growth was assigned as the MIC value.

Statistical analyses

For statistical comparison of biofilm formation or lactic acid accumulation in different groups, a two-sided t-test was used. Groups were considered statistically different if $p < 0.05$.

The OTU table was randomly subsampled at 3,400 reads/sample (the lowest number of reads/sample) and used for Shannon Diversity Index calculation in PAST software (19). The multivariate OTU dataset was $\log_2$

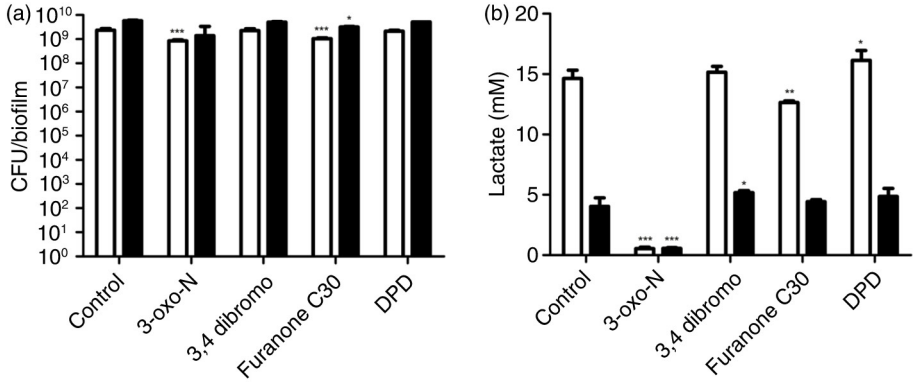

Fig. 1. Total biofilm formation and total lactic acid accumulation for biofilms grown in the presence of different quorum sensing (QS) inhibitors or precursors. a) Biofilm formation expressed as CFU per biofilm. b) Lactic acid accumulation in mM per biofilm after 3 h incubation in BPW containing 0.2% sucrose. For both panels, white bars represent 48-h biofilms; black bars represent 96-h biofilms. Statistical significance compared to the control is shown (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

transformed and ordinated by principal component analysis (PCA) into two dimensions using PAST.

Results

Quorum sensing modifier screening

Biofilm phenotypes, grown in the presence of different QS modifiers were compared after 48 and 96 h of growth. After 48 h, biofilm formation was only minimally affected by 3-Oxo-N and Furanone C30 (8.5×10^8 CFUs and 1.1×10^9 CFUs respectively, versus 2.2×10^9 CFUs for the control biofilms) (Fig. 1a). After 96 h of growth only Furanone C30 treatment resulted in statistically lower CFU counts. Although these differences were statistically significant, their biological relevance is minimal. All biofilms, even the significantly smaller ones, consisted of approximately 10^9 CFUs. In the oral cavity, differences in plaque formation smaller than one log most likely do not result in a clinically different outcome. As previously observed, 48-h biofilms produced significant amounts of lactic acid (9). Lactic acid accumulation was not or only slightly inhibited by addition of the QS modifiers, with the exception of 3-Oxo-N (Fig. 1b). 3-Oxo-N almost completely abolished lactic acid accumulation. This inhibitory effect of 3-Oxo-N was still observed after 96 h of growth.

Dose-dependent activity of 3-Oxo-N

Subsequently, a dose–response curve was determined for 3-Oxo-N on 48-h biofilms (Fig. 2a). Biofilm formation was not significantly affected by 3-Oxo-N, except for a slight yet significant inhibition of growth with 100 μM. In contrast, lactic acid accumulation was significantly reduced (16.3 compared to 10.1 mM) when 10 μM 3-Oxo-N was present during growth. Again, presence of 100 μM 3-Oxo-N resulted in biofilms with hardly any lactic acid accumulation.

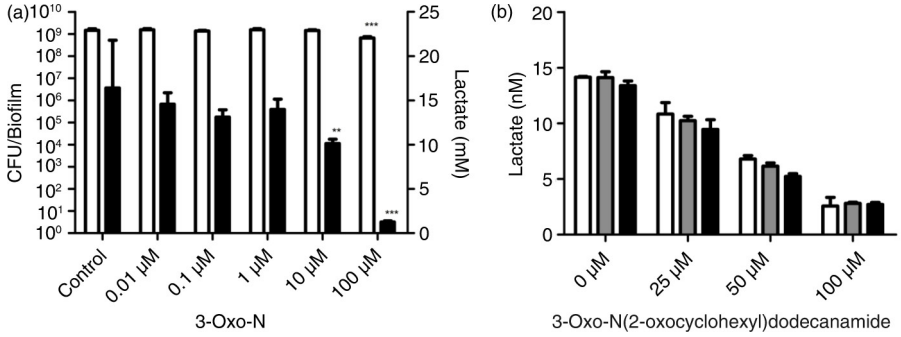

Fig. 2. Dose response of 3-Oxo-N and competition between 3-Oxo-N and C12-HSL. a) Total biofilm formation and total lactic acid accumulation for biofilms grown in the presence of different concentrations of 3-oxo-*N*-(2-oxocyclohexyl)dodecanamide. White bars: biofilm formation expressed as CFU/biofilm; black bars: lactic acid accumulation expressed in mM per biofilm after 3-h incubation in BPW containing 0.2% sucrose. Statistical significance compared to the control is shown (*$P<0.05$, **$P<0.01$, ***$P<0.001$). b) Total lactic acid accumulation for biofilms grown in presence of different concentrations 3-Oxo-N and C12-HSL, in mM per biofilm after 3-h incubation in BPW containing 0.2% sucrose. White bars: 0 μM C12-HSL; gray bars: 25 μM C12-HSL; black bars: 100 μM C12-HSL. All concentrations of 3-oxo-*N*-(2-oxocyclyhexyl)dodecanamide resulted in a statistical significant difference compared to the control.

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Competition of C12-HSL with 3-Oxo-N

3-Oxo-N is a homologue of the QS molecule C12-HSL and probably functions as a competitive inhibitor of C12-HSL detection in *P. aeruginosa* (10). Therefore, the effect of this natural QS molecule on the activity of 3-Oxo-N was evaluated (Fig. 2b). Increasing concentrations of C12-HSL did not affect lactic acid accumulation; in contrast, increasing concentrations of 3-Oxo-N again showed decreasing lactic acid accumulation. Addition of increasing concentrations of C12-HSL did not affect this decrease. These results suggest an alternative mechanism, different from QS interference, for inhibition of lactic acid accumulation by 3-Oxo-N.

Effect of 3-Oxo-N on the microbiome

In an attempt to explain the observed inhibition of lactic acid accumulation by 3-Oxo-N, its effect on the microbiome was determined (for full OTU table, see Supplementary Table 1). The inoculum contained 107 OTUs, while *in vitro* biofilm samples had on average 30.6 (SD 5.5) OTUs/sample. The Shannon Diversity Index was highest in the inoculum (3.0), followed by 96-h old biofilms and 48-h 3-Oxo-N-exposed biofilms (1.8), while 48-h control biofilms had the lowest diversity (1.4).

According to the PCA, the biofilm replicates were highly similar (Fig. 3a). The first component of the PCA, explaining 47.3% of the variance, separated the 96-h control biofilms from the others. Biofilms on the left side of the plot contained more *Haemophilus*/*Actinobacillus* (OTU142) while the 96-h control biofilms (right side) contained more *Prevotella* (OTU126, OTU240, OTU020), *Megasphaera* (OTU276), *Solobacterium* (OTU155) and *Alloprevotella* (OTU115). The 3-Oxo-N biofilms were located below their respective control biofilms in the PCA plot. The second component of the PCA, explaining 25.5% variance, was responsible for this separation. The control biofilms contained more *Veillonella* (OTU023), *Streptococcus* (OTU019, OTU198) and *Aggregatibacter* (OTU090) than their age-matched counterpart, while biofilms grown in the presence of 3-Oxo-N contained more *Campylobacter* (OTU097) and *Streptococcus* (OTU054).

Separate PCA plots per biofilm age (Fig. 3b and c) gave a clearer separation between the control biofilms and the 3-Oxo-N treated biofilms. For both analyses, the first

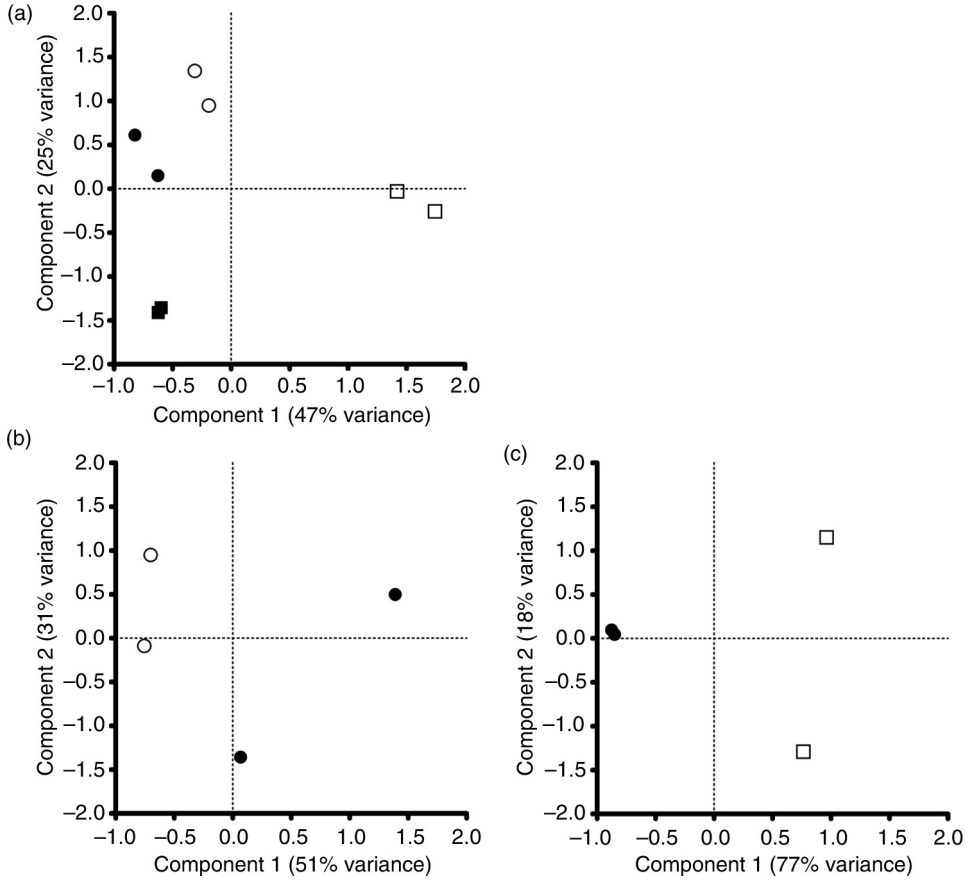

Fig. 3. Principal component analysis plot of a) all biofilms, b) 48-h biofilms, and c) 96-h biofilms, where ● are the 48-h biofilms grown in presence of 3-Oxo-N, ○ are the 48-h control biofilms, ■ are the 96-h biofilms grown in presence of 3-Oxo-N, □ and are the 96-h control biofilms. The data were randomly subsampled and $\log_2$-transformed.

principal component was responsible for this separation. In addition to the previously defined OTUs, *Prevotella* (OTU020) was more abundant in the control biofilms, while the 3-Oxo-N biofilms contained more *Haemophilus*/*Actinobacillus* (OTU142).

Genus-level analysis of the biofilm composition showed that growth for 48 h in the presence of 100 μM 3-Oxo-N induced a large decrease in relative abundance of *Streptococcus* spp. (33% in treated compared to 61% in control), a small increase in *Veillonella* spp. (36% in treated compared to 31% in control) and a strong increase in *Actinobacillus* spp. (29% in treated compared to 5% in control) (Fig. 4a and b). These differences remained after 96 h of growth (Fig. 4c and d).

Minimal inhibitory concentration of 3-Oxo-N on Streptococcus *spp.*

Microbiome analysis revealed a decrease in presence of streptococci in 3-Oxo-N grown biofilms. Streptococci are well known lactic acid producing, aciduric bacteria, often associated with caries (20). Decrease in their presence, related to species-specific toxicity of 3-Oxo-N, could explain the observed reduced lactic acid accumulation. The MIC of 3-Oxo-N for a panel of different oral *Streptococcus* spp. in pure culture ranged from 8 to 32 μM (Supplementary Table 2). Although there was a 4-fold difference in the MIC of the different *Streptococcus* species tested, all MIC values were within the active range of the compound.

Discussion and conclusion

Within the complex polymicrobial communities present in the oral cavity, intra- and interspecies communication mediated through QS systems is widespread. However, use of QS modifying approaches designed to maintain oral health is limited. In the present study, we aimed to investigate the effect of QS inhibitors on cariogenic dental plaque. For this purpose, we evaluated the effect of several QS modifiers on growth and cariogenic potential of *in vitro* oral biofilms. We evaluated the effect of the widespread AI-2 signal and two inhibitors thereof (Furanone C30 and 3,4-dibromo) (11, 12). In addition, we evaluated an inhibitor for the more species-specific C12-HSL signal (3-Oxo-N) (10). Of all tested compounds, addition of 3-Oxo-N resulted in the most striking effect. It inhibited the cariogenic potential *in vitro* without inducing a relevant inhibition of biofilm formation. Microbiome analysis showed a significant ecological change, possibly through species-selective growth inhibition. Although the exact mechanism remains unclear, it is unlikely that inhibition of C12-HSL-mediated communication is responsible for the observed effect.

Lack of biofilm growth inhibition by the test compounds was unexpected. Previous studies, using monocultures biofilms, describe impaired biofilm formation by QS inhibition in common oral bacteria (21, 22). Absence of inhibition in the present study might be related to the use of microcosm and illustrates the necessity of using more realistic oral biofilm models to predict effectiveness of novel prevention and treatment strategies.

Lactic acid is rapidly produced by carbohydrate-consuming, acidogenic bacteria, and this causes a severe drop in the local pH (23) resulting in demineralization of the tooth-surface. Therefore, the accumulation of lactic acid is a phenotype belonging to cariogenic plaque. Ergo, low lactic acid accumulation is indicative for a non-cariogenic biofilm. Growth in the presence of 10 μM 3-Oxo-N significantly decreased lactic acid accumulation compared to the control and presence 100 μM 3-Oxo-N resulted in a barely detectable lactic acid accumulation. Based on this *in vitro* activity, 3-Oxo-N could represent a novel compound used to prevent caries.

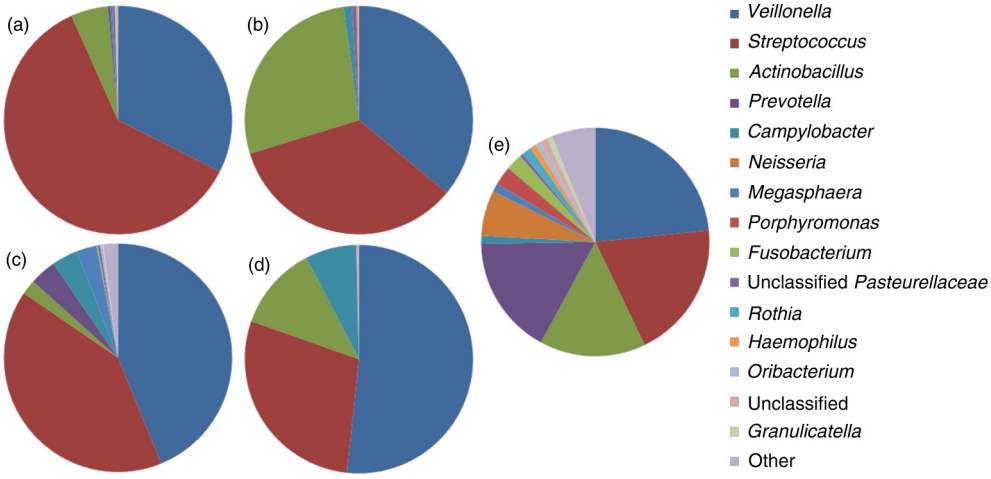

Fig. 4. Relative abundance of the top 15 most abundant genera (remaining genera are grouped as 'other') in: a) 48-h control biofilms, b) 48-h biofilms grown in presence of 3-Oxo-N, c) 96-h control biofilms, d) 96-h biofilms grown in presence of 3-Oxo-N and e) saliva inoculum.

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In general it is assumed that saccharolytic bacteria, mostly streptococci, are involved in rapid lactic acid production from sucrose. Microbiome analysis showed that 3-Oxo-N biofilms contained less *S. salivarius*/*S. vestibularis* compared to the control biofilms. Although these streptococcal species are not associated with dental caries, *S. vestibularis* has a mild cariogenic potential (24). Streptococci in general are acidogenic (20) and the 2-fold reduction in streptococci can explain the observed decreased cariogenic potential of 3-Oxo-N grown biofilms. Lack of *Prevotella* in biofilms grown in the presence of 3-Oxo-N is an additional indication of a less cariogenic ecology. Although *Prevotella* is not known for its acid production, it is often associated with caries lesions (25, 26).

The main carbon source of veillonellae is organic acids and preferably lactic acid (27). Because of their lactic acid consumption, and consequent pH-rise effect, they are considered to be beneficial bacteria in the oral cavity (28), and reduce the risk for caries. However, they are associated with caries lesions, most likely because of the availability of their preferred carbon source (29), rather as causative microorganisms in the caries process. Although their exact role in caries is not clear (30), the slightly higher relative abundance of veillonellae in 3-Oxo-N biofilms can be an additional reason for the reduction in the cariogenic phenotype of these biofilms. The combination of less streptococci and slightly more veillonellae result in a balanced metabolism where all the produced lactic acid is consumed, resulting in low lactic acid accumulation capacity of the biofilms.

However, 3-Oxo-N could have effects that go further than caries prevention. Microbiome analysis indicates that the presence of 3-Oxo-N during biofilm formation significantly affected species composition. Control biofilms cultured for 96 h contained *Megasphaera micronuciformis* and *Solobacterium moorei*. *Megasphaera* is associated with periodontal disease (31) and *S. moorei* is associated with halitosis, and was previously found in periodontal sites (32, 33). The complete absence of these species in biofilms grown in the presence of 3-Oxo-N suggests that the compound suppresses maturation and development of an unhealthy ecology. One exception is the increase in abundance of Campylobacter in biofilms grown in the presence of 3-Oxo-N, which is associated with periodontitis (34). Future studies are required to elucidate beneficial effects of 3-Oxo-N on periodontal biofilms.

The observed ecological changes cannot be explained by QS-inhibiting activity, as there was no competition between the inhibitor 3-Oxo-N and the natural QS molecule C12-HSL. C12-HSL (5-ring lactone) is a structural homologue of 3-Oxo-N (6-ring lactone). It is remarkable that no interaction could be observed between both molecules. C12-HSL did not induce or inhibit lactic acid accumulation, nor did it influence the inhibiting action of 3-Oxo-N. This selectivity could indicate the involvement of a specific binding protein with selectivity towards 6-ring lactone. Planktonic growth of several streptococci was inhibited by 3-Oxo-N; it is therefore possible that species-selective growth inhibition is involved in the ecological change and concomitant decreased cariogenic potential of the microcosm biofilms. Further studies are required to reveal the exact molecular mechanism of 3-Oxo-N.

Nowadays, the most effective remedies to prevent caries are sufficient oral hygiene levels (regular removal of the oral biofilm) in combination with a limited frequency of fermentable sugar consumption (35). In oral hygiene products, there are currently two effective compounds for caries prevention. Firstly, fluoride inhibits demineralization and promotes remineralization of the teeth, and after incorporation into the enamel, the enamel is slightly more resistant to future acid challenges (36). In addition, fluoride has been shown to reduce lactate production *in vivo* by inhibiting bacterial enzymes (37). Secondly, arginine is metabolized by oral bacteria resulting in increased pH and thus a less cariogenic environment (38). These compounds do not reduce acid production by oral bacteria, but the negative effects of the acid are reduced. Use of 3-Oxo-N in dentifrices could represent a third, fundamentally different approach. Several studies will be required to assess the clinical, *in vivo*, merits of 3-Oxo-N. Questions related to dose, exposure time, substantivity and frequency of administration are presently unanswered. 3-Oxo-N prevents acid accumulation by maintaining a non-cariogenic ecology in *in vitro* oral biofilm even in the presence of fermentable sugars and thus represents a promising compound in maintaining a healthy ecology in dental plaque.

Disclosure statement

The authors have declared that no competing interests exist.

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Conflict of interest and funding

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References

1. WHO (2012). World Health Organization: oral health fact sheet N °318. WHO Media Centre. Available from: http://www.who.int/mediacentre/factsheets/fs318/en/
2. Xavier KB, Bassler BL. LuxS quorum sensing: more than just a numbers game. Curr Opin Microbiol 2003; 6: 191–7.
3. Federle MJ, Bassler BL. Interspecies communication in bacteria. J Clin Invest 2003; 112: 1291–9.
4. McNab R, Ford SK, El-Sabaeny A, Barbieri B, Cook GS, Lamont RJ. LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. J Bacteriol 2003; 185: 274–84.
5. Hirano T, Beck DA, Demuth DR, Hackett M, Lamont RJ. Deep sequencing of *Porphyromonas gingivalis* and comparative transcriptome analysis of a LuxS mutant. Front Cell Infect Microbiol 2012; 2: 79.
6. Jarosz LM, Ovchinnikova ES, Meijler MM, Krom BP. Microbial spy games and host response: roles of a *Pseudomonas aeruginosa* small molecule in communication with other species. PLoS Pathog 2011; 7: e1002312.
7. Bachtiar EW, Bachtiar BM, Jarosz LM, Amir LR, Sunarto H, Ganin H, et al. AI-2 of *Aggregatibacter actinomycetemcomitans* inhibits Candida albicans biofilm formation. Front Cell Infect Microbiol 2014; 4: 94.
8. Glucksam-Galnoy Y, Sananes R, Silberstein N, Krief P, Kravchenko VV, Meijler MM, et al. The bacterial quorum-sensing signal molecule N-3-oxo-dodecanoyl-L-homoserine lactone reciprocally modulates pro- and anti-inflammatory cytokines in activated macrophages. J Immunol 2013; 191: 337–44.
9. Janus MM, Keijser BJ, Bikker FJ, Exterkate RA, Crielaard W, Krom BP. *In vitro* phenotypic differentiation towards commensal and pathogenic oral biofilms. Biofouling 2015; 31: 503–10.
10. Smith KM, Bu Y, Suga H. Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs. Chem Biol 2003; 10: 81–9.
11. He Z, Wang Q, Hu Y, Liang J, Jiang Y, Ma R, et al. Use of the quorum sensing inhibitor furanone C-30 to interfere with biofilm formation by *Streptococcus mutans* and its luxS mutant strain. Int J Antimicrob Agents 2012; 40: 30–5.
12. Zang T, Lee BW, Cannon LM, Ritter KA, Dai S, Ren D, et al. A naturally occurring brominated furanone covalently modifies and inactivates LuxS. Bioorg Med Chem Lett 2009; 19: 6200–4.
13. Exterkate RA, Crielaard W, Ten Cate JM. Different response to amine fluoride by *Streptococcus mutans* and polymicrobial biofilms in a novel high-throughput active attachment model. Caries Res 2010; 44: 372–9.
14. van Loveren C, Buijs JF, ten Cate JM. The effect of triclosan toothpaste on enamel demineralization in a bacterial demineralization model. J Antimicrob Chemother 2000; 45: 153–8.
15. O'Donnell LE, Robertson D, Nile CJ, Cross LJ, Riggio M, Sherriff A, et al. The oral microbiome of denture wearers is influenced by levels of natural dentition. PLoS One 2015; 10: e0137717.
16. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009; 75: 7537–41.
17. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007; 73: 5261–7.
18. Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database (Oxford) 2010; 2010: baq013.
19. Hammer Ø, Harper D, Ryan P. PAST–PAlaeontological STatistics, ver. 1.89. Palaeontol Electron 2001; 4: 1–9.
20. De Soet JJ, Toors FA, De Graaff J. Acidogenesis by oral streptococci at different pH values. Caries Res 1989; 23: 14–7.
21. Jang YJ, Choi YJ, Lee SH, Jun HK, Choi BK. Autoinducer 2 of *Fusobacterium nucleatum* as a target molecule to inhibit biofilm formation of periodontopathogens. Arch Oral Biol 2013; 58: 17–27.
22. Merritt J, Qi F, Goodman SD, Anderson MH, Shi W. Mutation of luxS affects biofilm formation in *Streptococcus mutans*. Infect Immun 2003; 71: 1972–9.
23. Matsui R, Cvitkovitch D. Acid tolerance mechanisms utilized by *Streptococcus mutans*. Future Microbiol 2010; 5: 403–17.
24. Chestnutt IG, MacFarlane TW, Stephen KW. An *in vitro* investigation of the cariogenic potential of oral *Streptococci*. Arch Oral Biol 1994; 39: 589–93.
25. Johansson I, Witkowska E, Kaveh B, Lif Holgerson P, Tanner AC. The microbiome in populations with a low and high prevalence of caries. J Dent Res 2016; 95: 80–6.
26. Nadkarni MA, Caldon CE, Chhour KL, Fisher IP, Martin FE, Jacques NA, et al. Carious dentine provides a habitat for a complex array of novel *Prevotella*-like bacteria. J Clin Microbiol 2004; 42: 5238–44.
27. Distler W, Kroncke A. The lactate metabolism of the oral bacterium *Veillonella* from human saliva. Arch Oral Biol 1981; 26: 657–61.
28. Van der Hoeven JS, Toorop AI, Mikx RH. Symbiotic relationship of *Veillonella alcalescens* and *Streptococcus mutans* in dental plaque in gnotobiotic rats. Caries Res 1978; 12: 142–7.
29. Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994; 8: 263–71.
30. Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL. Beyond *Streptococcus mutans*: dental caries onset linked to multiple species by 16S rRNA community analysis. PLoS One 2012; 7: e47722.
31. Kumar PS, Griffen AL, Moeschberger ML, Leys EJ. Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. J Clin Microbiol 2005; 43: 3944–55.
32. Colombo AP, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, et al. Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. J Periodontol 2009; 80: 1421–32.
33. Vancauwenberghe F, Dadamio J, Laleman I, Van Tornout M, Teughels W, Coucke W, et al. The role of *Solobacterium moorei* in oral malodour. J Breath Res 2013; 7: 046006.
34. Macuch PJ, Tanner AC. *Campylobacter* species in health, gingivitis, and periodontitis. J Dent Res 2000; 79: 785–92.
35. Rugg-Gunn A. Dental caries: strategies to control this preventable disease. Acta Med Acad 2013; 42: 117–30.
36. Ten Cate JM. Current concepts on the theories of the mechanism of action of fluoride. Acta Odontol Scand 1999; 57: 325–9.
37. Takahashi N, Washio J. Metabolomic effects of xylitol and fluoride on plaque biofilm *in vivo*. J Dent Res 2011; 90: 1463–8.
38. Nascimento MM, Browngardt C, Xiaohui X, Klepac-Ceraj V, Paster BJ, Burne RA. The effect of arginine on oral biofilm communities. Mol Oral Microbiol 2014; 29: 45–54.