Aggregatibacter actinomycetemcomitans mediates protection of Porphyromonas gingivalis from Streptococcus sanguinis hydrogen peroxide production in multi-species biofilms

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Mixed species biofilms are shaped and influenced by interactions between species. In the oral cavity, dysbiosis of the microbiome leads to diseases such as periodontitis. Porphyromonas gingivalis is a keystone pathogen of periodontitis. In this study, we showed that polymicrobial biofilm formation promoted the tolerance of Porphyromonas gingivalis to oxidative stress under micro-aerobic conditions. The presence of Streptococcus sanguinis, an oral commensal bacterium, inhibited the survival of P. gingivalis in dual-species biofilms via the secretion of hydrogen peroxide (H₂O₂). Interestingly, this repression could be attenuated by the presence of Aggregatibacter actinomycetemcomitans in tri-species biofilms. It was also shown that the katA gene, encoding a cytoplasmic catalase in A. actinomycetemcomitans, was responsible for the reduction of H₂O₂ produced by S. sanguinis, which consequently increased the biomass of P. gingivalis in tri-species biofilms. Collectively, these findings reveal that polymicrobial interactions play important roles in shaping bacterial community in biofilm. The existence of catalase producers may support the colonization of pathogens vulnerable to H₂O₂ in the oral cavity. The catalase may be a potential drug target to aid in the prevention of periodontitis.

According to the 2016 global burden of disease study, periodontal disease is estimated to affect 750,847 million people worldwide, making it the 11th most prevalent human disease. It is an inflammatory disorder, characterized by the destruction of tooth-supporting tissues such as gingiva, periodontal ligament and alveolar bone. Periodontitis is caused by the dysbiosis of the oral microbiome. In the pathogenesis of periodontitis, the subgingival microbiome switches from majority Gram-positive to majority Gram-negative bacterial species. Porphyromonas gingivalis is a Gram-negative bacterium which is regarded as one of the keystone pathogens in chronic periodontitis. It produces virulence factors to disrupt host–microbial homeostasis, resulting in inflammation and bone loss. Oral microbiome studies by 16s rRNA sequencing suggest that the abundance of periodontitis-associated species, such as P. gingivalis, Treponema denticola and Tannerella forsythia, is significantly increased in disease sites of periodontitis patients. Many of these pathogens are anaerobic species that survive in deep dental pockets where oxygen is limited. Surprisingly, these anaerobic pathogens, including P. gingivalis, have also been reported in supragingival plaque, saliva and mucosa samples which are thought to be more micro-aerobic environments in the oral cavity. Several proteins have been reported to participate in the resistance to oxidative stress in P. gingivalis and they may promote the survival of P. gingivalis under micro-aerobic conditions.

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The oxidative stress in the oral microbiome is not only related to oxygen concentration in the surroundings but reactive oxygen species (ROS) produced by eukaryotic cells and some oral commensal bacteria\(^{13-17}\). ROS leads to protein, DNA, and lipid damage, and results in an increased rate of mutagenesis and cell death\(^{18}\). *Streptococcus gordonii* is a Gram-positive, facultative anaerobe bacterium that is able to inhibit the growth of *P. gingivalis*\(^{19}\) and produce a ROS, hydrogen peroxide (H\(_2\)O\(_2\))\(^{19,20}\). It is a pioneering colonizer in the oral cavity and a key player in oral biofilm development\(^{21,22}\). Because the abundance was significantly decreased in the diseased subgingival microbiome, *S. sanguinis* was thought to be an oral health-associated species\(^{23,24}\). It is feasible that *S. sanguinis* maintains a healthy oral homeostasis by generating H\(_2\)O\(_2\) in the oral cavity. Several genes have been reported to be responsible for H\(_2\)O\(_2\) production in oral microbiota.

**Results**

**The impact of *S. sanguinis* and *A. actinomycetemcomitans* on the biomass of *P. gingivalis* in multi-species biofilms.** It has been reported that *P. gingivalis*, are many anaerobic species, including *P. gingivalis*, are widely distributed in the oral cavity\(^{25-28}\). We performed experiments to evaluate the survival of *P. gingivalis* in 3 environments: 14 mL test tubes with shaking at 100 rpm under micro-aerobic conditions (6% oxygen, gas mixture), and 4-well chambers without shaking (static) under either anaerobic (0% oxygen, gas mixture) or micro-aerobic conditions. The incubator shaking was used to inhibit biofilm growth and thus encourage planktonic growth while the lack of shaking was used to facilitate biofilm formation. All three environments had an initial inoculation of 1 × 10\(^8\) *P. gingivalis* ATCC 33277 (Pg) cells into CDM and were incubated for four days at 37\(^\circ\)C. When *Pg* was cultured in the 4 mL tubes environment, it was not able to survive (Fig. 1A). *Pg* survived in both 4-well chamber environments; the biomass under micro-aerobic conditions was significantly lower than that of the initial inoculation, it was still greater than the CFU of *Pg* grown in static micro-aerobic conditions (Fig. 1A). Although the CFU of *Pg* grown under micro-aerobic conditions in 4-well chambers was lower than that of the initial inoculation, it was still greater than the CFU of *Pg* grown in the 14 mL test tube, suggesting that the biofilm formation increased the tolerance of *Pg* to oxidative stress from the presence of environmental oxygen (Fig. 1A). Because there have already been a number of reports illustrating that biofilm formation increase the tolerance of bacteria to oxidative stress\(^{33}\), the next experiment was designed to focus on the effect of mixed-species biofilm bacterial interactions on the oxidative stress tolerance of *P. gingivalis*.

To examine *Pg* survival in multi-species biofilms, four groups of bacterial mixes were tested: *Pg* only, *Pg* and *A. actinomycetemcomitans* 652 (Aa), *Pg* and *S. sanguinis* SK36 (Ss) and a mixture of all three species. Each bacterial mix group was incubated in CDM medium under micro-aerobic conditions in 4-well chambers. Biofilms were first grown for four days, after which they were stained by fluorescence in situ hybridization (FISH) and were visualized using confocal laser scanning microscopy (CLSM). Biofilm biomass was quantified by COMSTAT script in Matlab software\(^{34}\).

All four groups of biofilms were successfully detected using FISH probes. Under these micro-aerobic conditions, the biomass of *Pg* in biofilm was not significantly changed by the presence of *Aa* when compared to the *Pg* single species biofilm control (Fig. 1B.C). In contrast, the presence of *Ss* significantly lowered the biomass of *Pg* in *Ss-Pg* dual-species biofilms, suggesting that *Ss* was dominant and somehow inhibited survival of *Pg* (0.442 ± 0.083 \(\mu m^3/\mu m^2\) in *Pg* only biofilm and 0.021 ± 0.009 \(\mu m^3/\mu m^2\) in *Pg-Ss* dual species biofilm (P < 0.001)) (Fig. 1B.C). Interestingly, the biomass of *Pg* in *Pg-Aa-Ss* tri-species biofilm was significantly increased compared to that in *Pg-Ss* dual-species biofilms, implying that *Pg* survival inhibition by *Ss* could be partially attenuated by the presence of *Aa* (Fig. 1B.C). As there was no significant difference between the biomass of *Pg* from *Pg* single species...
H₂O₂ might affect inter-species attachment. It has been shown that the concentration of H₂O₂ in wild type (WT), the ΔSs mutant could still produce about 25% the H₂O₂ produced by WT biofilm, which indicated that H₂O₂ production in Ss-ΔspxB mutant was decreased (Fig. 3A). This result suggested that H₂O₂ was essential for P. gingivalis survival in multi-species biofilms, which was similar to the interaction between P. gingivalis and S. gordonii35.

Since more Pg survived in the Pg-Aa-Ss tri-species biofilm, the supplementation of Aa might have a better effect than the deletion of spxB on reducing H₂O₂ concentration. To test this hypothesis, Ss WT and Ss ΔspxB dual-species biofilms with/without the addition of Aa were cultured. The amount of H₂O₂ in the supernatant of the 4-day old biofilms was measured using Hydrogen Peroxide Assay. Indeed, the H₂O₂ concentration in the Aa-Ss WT dual-species biofilm was much lower than that in the Ss ΔspxB single species biofilm (P ≤ 0.0001), which supported the hypothesis (Fig. 3A). Additionally, in comparison to the WT biofilm, the Ss ΔspxB single species biofilm contained less H₂O₂ in the supernatant (P ≤ 0.0001), which was consistent with the result in the previous study showing that the spxB gene deletion decreased H₂O₂ production in Ss (Fig. 3A)19.

When Pg was co-cultured with Ss ΔspxB, the biomass of Pg was still greatly inhibited by Ss ΔspxB (Fig. 2B). One probability was that, in contrast with Ss wild type (WT), the ΔspxB mutant could still produce about 25% the concentration of H₂O₂, which might be enough for the inhibition of Pg growth.

SSS-produced H₂O₂ reduced the biomass of Pg. H₂O₂ is a well-studied inhibitory mechanism that S. sanguinis uses to compete with Streptococcus mutans35,36. It can be generated by a pyruvate oxidase (SpxB) in Ss via a reaction converting pyruvate to acetyl phosphate. During this catalytic process, oxygen is consumed35,36. During this process, S. sanguinis might coaggregate with P. gingivalis, indirectly promoting the survival of P. gingivalis.

The Aa-Ss ΔspxB dual-species biofilm contained less H₂O₂ than the Aa-Ss WT biofilm, which indicated that the spxB gene deletion might promote Pg survival in Pg-Aa-Ss tri-species biofilms (Fig. 3A). 4-day old Pg-Aa-Ss WT and Pg-Aa-Ss ΔspxB tri-species biofilms were treated with FISH and observed by CLSM. The biomass of Pg in the Pg-Aa-Ss ΔspxB tri-species biofilm was more than that in the Pg-Aa-Ss WT biofilm (P ≤ 0.01), which suggested that the H₂O₂ produced by Ss played an important role in inhibiting Pg growth (Fig. 3B,C).

Similar to Pg, the biomass of Aa was also increased in the Pg-Aa-Ss ΔspxB biofilm (P ≤ 0.001) (Fig. 3B,C). Surprisingly, the biomass of Strep. S. mutans was increased in the Pg-Aa-Ss ΔspxB biofilms than that in the Pg-Aa-Ss WT tri-species biofilm (P ≤ 0.05) (Fig. 3B,C), despite Ss ΔspxB had a reduced biofilm formation in Ss-Pg dual-species biofilms (Figs 2B and S2). When we treated tri-species biofilms using FISH protocol, we observed that the Pg-Aa-Ss WT biofilm was more fragile than the Pg-Aa-Ss ΔspxB biofilm, indicating that H₂O₂ might affect inter-species attachment. It has been shown that P. gingivalis utilizes fimbrillin to bind to glyceraldehyde-3-phosphate dehydrogenase, a cell surface protein of S. sanguinis37, implying that S. sanguinis may not only inhibit the growth of P. gingivalis but also coaggregate with P. gingivalis. Due to the deletion of spxB, a reduced antagonism in Pg-Aa-Ss tri-species biofilm might be beneficial for the co-aggregation between Pg and Aa.
Ss, and as a result, might increase the biomass of Ss. Though a similar relationship may exist between S. sanguinis and A. actinomycetemcomitans, the current knowledge on their interactions is limited, and the mechanism of such phenomenon needs further exploration.

**Aa degraded H₂O₂ and protected Pg from H₂O₂ attack.** The data in Fig. 3A implied that Aa might impact Ss and indirectly promote the survival of Pg through degrading H₂O₂. To further elucidate whether Aa protected Pg from H₂O₂, the concentration of H₂O₂ was measured using Hydrogen Peroxide Assay. Briefly, cells were resuspended in fresh CDM and mixed with Hydrogen Peroxide Assay solution. The final reaction solutions were incubated under room atmospheric conditions at 37 °C. The optical density for cell growth and fluorescent signal for H₂O₂ concentration were monitored by the plate reader.

Firstly, the Hydrogen Peroxide Assay solution was supplemented with H₂O₂ (2, 1.5 or 1 μM). It was mixed or not mixed with Aa suspension. The H₂O₂ concentration was recorded after 30 minutes of reaction. The presence of Aa greatly reduced H₂O₂ concentrations for all variants of the Hydrogen Peroxide Assay solution, which demonstrated that Aa has the ability to degrade H₂O₂ (Fig. 4A).

Subsequently, the H₂O₂ produced by Ss, Pg and Aa was tested. Compared with the blank control (CDM without bacteria), neither Pg nor Aa produced H₂O₂ (Fig. 4B). The H₂O₂ concentration in Aa was even lower than the concentration in the blank control (Fig. 4B). Ss produced almost 0.3 μM of H₂O₂ after 30 minutes of reaction, which could be attenuated by the addition of Aa (P ≤ 0.001) but not Pg (Fig. 4B). The presence of Aa decreased the H₂O₂ concentration by nearly half in both the dual-species (Ss-Aa) and tri-species (SS-Pg-Aa) suspensions (Fig. 4B). There was no significant change in cell density during the 30 minutes of experimentation, indicating that cell growth did not influence the results of H₂O₂ concentrations (Fig. S3). Similar results were reported in previous works, which utilized scanning electrochemical microscopy to do real-time mapping of H₂O₂ concentrations on bacteria biofilms. They reported that the H₂O₂ generated by S. gordonii, another oral commensal
The bacterium and H$_2$O$_2$ producer, could be reduced by A. actinomycetemcomitans$^{38}$. These data suggested that Aa degraded H$_2$O$_2$ produced by Ss and implied that Aa might be able to promote the survival of Pg in Pg-Aa-Ss tri-species biofilm by reducing H$_2$O$_2$ concentration.

Bacteria living in biofilms are surrounded by matrix composed of polysaccharide, eDNA and proteins$^{39}$. As materials may slow penetrate and transverse a biofilm$^{39}$, cell to cell distance may impact the interaction between Aa and Ss. To test the contribution of cell-cell distance to the Aa–Ss interaction, Aa and Ss were cultured in a transwell system, where they were separated by a 0.4 μm filter. Ss was cultured at the bottom and Aa was either incubated in the insert or mixed with Ss at the bottom. After 30 minutes of reaction, the H$_2$O$_2$ concentration at the bottom was measured. The H$_2$O$_2$ concentration at the bottom was 2.404 ± 0.035 μM when the insert was filled with CDM medium and the bottom was Ss. When Aa was put in the insert and Ss was set at the bottom of the well, Aa slightly but significantly decreased the H$_2$O$_2$ concentration at the bottom to 2.087 ± 0.061 μM (P ≤ 0.05) (Fig. 4C). However, the reduction was much lower than that in the well where Ss and Aa mixed directly at the bottom (P ≤ 0.001) (Fig. 4C). This result showed that a closer distance between Ss and Aa was beneficial for Aa to reduce H$_2$O$_2$ produced by Ss. Aa might have limited function to degrade H$_2$O$_2$ when it was far away from Ss. In an in vitro study, Aggregatibacter has been shown to close contact with Streptococcus$^{40}$, indicating that A. actinomycetemcomitans might exist near to S. sanguinis in vivo to detoxify H$_2$O$_2$.  

Figure 3. The effect of the spxB gene deletion on Pg biomass in Pg-Aa-Ss tri-species biofilms under micro-aerobic conditions. (A) Ss WT and Ss ΔspxB biofilms with/without the supplement of Aa were cultured. The H$_2$O$_2$ concentration in the supernatant of these biofilms was measured by the Hydrogen Peroxide Assay as described in Materials and methods. (B) Pg-Aa-Ss WT (left) and Pg-Aa-Ss ΔspxB tri-species (right) (Pg = green, Ss = blue, Aa = red) biofilms were shown. (C) The biomass of Pg, Aa and Ss in B was quantified by COMSTAT and shown as a bar chart. Scale bars were indicated on the corresponding images. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, Student’s t-test. Means and standard deviations from triplicate experiments are shown.
Phagology of ΔkatA Pg have been impacted by A. actinomycetemcomitans and/or to the observed phenomenon in Fig. 3B,C allowing for the possible hypothesis that the biomass of Ss Aa biomass in Aa film biomass of these two strains were similar, which confirmed that the reduction of (Fig. S5A). It contained larger aggregations and bigger gaps between aggregations (Fig. S5A). However, the bio-
detoxify H2O2 and is essential for the survival of Pg. P* A. actinomycetemcomitans further confirmed the hypothesis that comitans H2O2 concentrations of Figure 5.

Figure 4. The effect of Aa on the concentration of H2O2. (A) Aa suspensions were mixed with H2O2 solutions. H2O2 concentrations were then tested after 30 minutes of reaction. (B) H2O2 concentrations of different bacterial mixtures were measured at 10-minute intervals. The enlarged section of the graph, illustrated as a bar chart, showed the H2O2 concentration after 30 minutes of reaction. CDM media without bacteria was used as a blank control. (C) Wells were divided into two parts by a transwell system. Ss, Aa or CDM medium were located at different places as shown in the figure. The H2O2 concentration in the bottom section was measured. *P ≤ 0.05, ***P ≤ 0.001, Student’s t-test. Means and standard deviations from triplicate experiments are shown.

Using the Hydrogen Peroxide Assay Kit, H2O2 concentration and cell density of Pg, Aa VT1169 and Ss were recorded at 10-minute intervals using the Hydrogen Peroxide Assay. (B) The biomass of Pg, Ss, Aa VT1169 and Pg-Ss-Aa ΔkatA were stained by FISH and observed by CLSM. Pg, Aa VT1169 and Ss were marked as green, red and blue, respectively. (C) The biomass of Pg, Aa VT1169 and Ss in (B) were quantified by COMSTAT. Scale bars were indicated on the corresponding images. *P ≤ 0.05, ***P < 0.001, Student’s t-test. Means and standard deviations from triplicate experiments are shown.

KatA has been reported to produce catalase in A. actinomycetemcomitans strain VT1169 (Aa VT1169) to detoxify H2O2 and is essential for the survival of A. actinomycetemcomitans during co-infection with S. gordonii[24,25]. It was hypothesized that KatA was essential for Aa to improve the survival of Pg.

Using the Hydrogen Peroxide Assay Kit, H2O2 concentration and cell density of Pg + Ss + Aa VT1169 and Pg + Ss + A. actinomycetemcomitans VT1169 ΔkatA (Aa ΔkatA) suspensions were monitored. Compared to the ΔkatA deletion mutant, Aa VT1169 had the greater ability to repress H2O2 production (P ≤ 0.001 at the time point of 110 minutes), implying that KatA was important for Aa to reduce the H2O2 generated by Ss (Fig. 5A). There was no significant difference in cell density between Pg + Ss + Aa VT1169 and Pg + Ss + Aa ΔkatA, suggesting that the difference in H2O2 concentration was not caused by a difference in cell growth (Fig. S4).

Pg-Ss-Aa VT1169 and Pg-Ss-Aa ΔkatA Tri-species biofilms were stained by FISH and observed by CLSM as described above. Compared with the biofilm of Pg-Ss-Aa VT1169, the Pg-Ss-Aa ΔkatA biofilm contained less Pg and Aa (P ≤ 0.001 for both comparisons) (Fig. 5B,C), which suggested that the catalase of A. actinomycetemcomitans was essential for the survival of both Pg and A. actinomycetemcomitans in the tri-species biofilms and further confirmed the hypothesis that A. actinomycetemcomitans protected P. gingivalis from H2O2 damage. The biofilm biomass of Ss in Pg-Ss-Aa ΔkatA was slightly decreased (P ≤ 0.05) (Fig. 5B,C). This phenomenon where Ss biofilm biomass decreased in conditions that also led to decreased biofilm biomass of Aa and Pg, was similar to the observed phenomenon in Fig. 3B,C allowing for the possible hypothesis that the biomass of Ss might have been impacted by Pg and/or A. actinomycetemcomitans in tri-species biofilms.

The VT1169 and ΔkatA single species biofilms were stained by SYTO9 and observed by CLSM. The morphology of ΔkatA biofilm was different from that of the wild type strain. The biofilm of ΔkatA was much thicker (Fig. S5A). It contained larger aggregations and bigger gaps between aggregations (Fig. S5A). However, the biofilm biomass of these two strains were similar, which confirmed that the reduction of Aa biomass in Pg-Ss-Aa ΔkatA tri-species was not caused by an attenuated biofilm formation ability of Aa ΔkatA (Fig. S5).

Figure 5. The influence of the katA gene of Aa VT1169 on Pg-Ss-Aa VT1169 tri-species biofilms. (A) The H2O2 concentrations of Pg-Ss-Aa VT1169 and Pg-Ss-Aa ΔkatA were recorded at 10-minute intervals using the Hydrogen Peroxide Assay. (B) The biomass of Pg, Ss, Aa VT1169 and Pg-Ss-Aa ΔkatA were stained by FISH and observed by CLSM. Pg, Aa VT1169 and Ss were marked as green, red and blue, respectively. (C) The biomass of Pg, Aa VT1169 and Ss in (B) were quantified by COMSTAT. Scale bars were indicated on the corresponding images. *P ≤ 0.05, ***P < 0.001, Student’s t-test. Means and standard deviations from triplicate experiments are shown.
Discussion
In this study, it was shown that *Aa* degraded H$_2$O$_2$ produced by *Ss*, which consequently aided the survival of *Pg* in *Pg*-*Aa*-*Ss* tri-species biofilms under micro-aerobic conditions. KatA, which produces catalase in *Aa*, was also shown to participate in this interaction. There have been many epidemiological studies showing that anaerobic bacteria such as *P. gingivalis* exist in supragingival, salivary and mucosal samples$^{4,10}$. One possibility is that micro-environment may exist in oral cavity, which allows these anaerobic bacteria to survive in micro-aerobic conditions. The results of this study presented the possibility that catalase producers in oral microbiota attenuate the oxidative stress and help the survival of anaerobic species under micro-aerobic conditions.

The pathogenesis of periodontitis has been thought to possess poly-microbial synergistic interactions$^{41,42}$. A temporal dynamics study showed that facultative anaerobic bacteria, especially *Streptococcus*, were dominant in the early stage of oral biofilm formation$^{41}$. Subsequently, the ‘healthy’ biofilm composition was replaced with a population of gram-negative anaerobic bacteria$^{43}$. In our study, we observed essentially no *Pg* presence in *Pg*-*Ss* dual species biofilms but *Pg* presence was obvious in *Pg*-*Aa*-*Ss* tri-species biofilms, suggesting that the existence of *Aa* was important for *Pg* survival. This result indicated that the earlier colonization of bacteria species with catalase activity than anaerobic species in oral biofilms might be necessary to generate suitable surroundings for the survival of anaerobic microorganisms. Further studies need to be performed to test this hypothesis. Additionally, our study illustrated that *KatA* of *Aa* VT1169 was important for the growth of both *Pg* and *Aa* VT1169, implying that catalase might be a promising drug target to prevent periodontitis.

Welch et al. utilized FISH technology to stain supragingival dental plaque$^{40}$. They hypothesized that the *Porphyromonas* growing at the periphery of biofilm samples might not be *P. gingivalis* because the outer shell of the biofilm was in a presumably aerobic environment$^{45}$. Here, we demonstrated that *P. gingivalis* was able to survive in a micro-aerobic environment and had better survival in the presence of *A. actinomycetemcomitans*, which implied that it was possible that the bacteria at the periphery of supragingival biofilm samples, seen in the Welch’s study, was *P. gingivalis*. In their study, they showed that both *Porphyromonas* and *Haemophilus/Aggregatibacter* were in close contact with *Streptococcus* cells$^{45}$. Furthermore, *Aggregatibacter* was not found adjacent to cells of *Porphyromonas* in the absence of *Streptococcus*$^{45}$. Their results indicated that *P. gingivalis, A. actinomycetemcomitans* and *S. sanguinis* might be close to each other in vivo and a similar interaction between these three species might also exist in vivo.

In Fig. 2A, *Pg* appeared to preferentially colocalize with *Ss* in *Pg*-*Ss* dual-species biofilms when catalase was supplemented. Additionally, the biomass of *Ss* in both Figs 3B and 5B were positively related with the biomass of *Pg* and *Aa*. All the phenomena above indicated that *Ss* might also cooperate with *Pg* and/or *Aa* in multi-species biofilms. The antagonism and the cooperation between commensal bacteria and pathogens may exist in equilibrium in oral microbiota. Whenever the antagonism was weakened, or the cooperation was strengthened either by other microorganisms or environmental conditions, dysbiosis may happen and lead to diseases such as periodontitis.

Materials and Methods

Bacterial strains, growth and antibiotics. Strains used in this study are listed in Table S1. Unless otherwise stated, *Pg, A. actinomycetemcomitans* strains and *Ss* cells from −80 °C frozen glycerol stocks were 0.5% inoculated into TSB medium (tryptic soy broth supplemented with yeast extract (5 mg/ml), hemin (5 μg/ml) and menadione (1 μg/ml)) and incubated statically under anaerobic conditions (10% CO$_2$, 10% H$_2$ and 80% N$_2$) at 37 °C using an Anoxomat® system (Spiral Biotech, Norwood, MA). Specinomycin was used at 50 μg/ml for the culture of *Aa ΔkatA*. No antibiotic was added to multi-species biofilms. The CFUs of *Pg* were tested by growing *Pg* on sheep blood agar plates (Trypticase® Soy Agar (TSA II™) with Sheep Blood, BD BBL™) under anaerobic conditions. All media was incubated in anaerobic jars for at least 2 days before experiments to equilibrate.

Biofilm assay. *Pg, A. actinomycetemcomitans* strains and *Ss* were initially incubated separately for 48 hours, 24 hours and overnight respectively, in TSB medium under anaerobic conditions to early stationary phase. The resultant growth was then resuspended in fresh CDM, followed by 10% inoculation into CDM medium and incubation under micro-aerobic conditions for biofilm formation. CDM was prepared as previously described (10.0 mM of NaH$_2$PO$_4$, 10.0 mM of KCl, 2.0 mM of citric acid, 1.25 mM of MgCl$_2$, 100.0 μM of FeCl$_3$, 20.0 μM of CaCl$_2$, 0.1 μM of Na$_2$MoO$_4$, 25.0 μM of ZnCl$_2$, 50.0 μM of MnCl$_2$, 5.0 μM of CuCl$_2$, 10.0 μM of CoCl$_2$, 5.0 μM of H$_2$BO$_3$, 1% (w/v) Tryptone, 7.67 μM of Hemin and 2.91 μM of Menadione)$^{44}$. Biofilms were incubated in 4-chambered glass coverslip wells (Chambered Coverglass, Nunc™ Lab-Tek™) for 4 days at 37 °C. Cultures were grown anaerobically (0% O$_2$, 10% CO$_2$, 10% H$_2$ and 80% N$_2$) or micro-aerobically (6% O$_2$, 7.2% CO$_2$, 7.2% H$_2$ and 79.6% N$_2$) in jars using the Anoxomat® system (Spiral Biotech, Norwood, MA). Single-species biofilms were stained using SYTO 9 (SYTO™ 9 Green Fluorescent Nucleic Acid Stain, Invitrogen™ Molecular Probes™) and FISH was used to analyze and characterize the composition in multi-species biofilms.

FISH assay. FISH was performed as previously described$^{46}$. FISH probes used in the study were ordered from Integrated DNA Technologies, Inc. and the sequences were listed in Table S2. Biofilms were grown in 4-well chambers for 4 days in 1 mL of CDM medium. 800 μL of supernatant was discarded by pipetting and then 4-well chambers were slowly turned over on paper towels to discard remaining supernatant. Biofilms were gently washed by 200 μL of 1× PBS buffer and fixed by 2% (wt/vol) paraformaldehyde on ice for at least 1.5 hours. After fixation, samples were then gently washed again in 1× PBS for 15 min. Next, PBS was discarded and 10 μL of hybridization solution (900 mM of NaCl, 20 mM of Tris, pH 7.5, 0.01% of SDS, 20% (vol/vol) of formamide, each probe at a final concentration of 0.1 μM) was dropped on biofilm samples and stained at 46 °C for 4 hours in a chamber humidified with 20% (vol/vol) formamide. Samples were then gently washed in wash buffer (215 mM of NaCl, 20 mM of Na$_2$HPO$_4$).
of Tris, pH 7.5, 5 mM of EDTA) at 48 °C for 15 minutes. Finally, biofilms were gently washed by cold water, and mounted in ProLong Gold Antifade Solution (ThermoFisher) for CLSM observation.

**CLSM and biomass quantification.** FISH-treated biofilms were observed by a Zeiss LSM 710 confocal laser scanning microscope (Zeiss, Germany) (VCU Core Facilities) and quantified by COMSTAT in Matlab software\(^3\). The fluorescent dyes were listed in Table S2. Three images of each sample were quantified to calculate the means and standard deviations.

**Hydrogen Peroxide Assay.** \(\text{H}_2\text{O}_2\) concentration was measured by Hydrogen Peroxide Assay Kit (Red Hydrogen Peroxide/Peroxidase Assay Kit, Amplex®\(^\text{TM}\)). Operations followed a standard protocol of the kit. For testing \(\text{H}_2\text{O}_2\) concentration in biofilm supernatant, 80 µl of biofilm supernatant was centrifuged. Subsequently, 50 µl of supernatant was mixed with Hydrogen Peroxide Assay solution. After 30 minutes of reaction, the fluorescent signal (excitation 560 nm/emission 590 nm) was recorded by a Synergy H1 Hybrid Reader. The preparation of a standard curve for quantifying \(\text{H}_2\text{O}_2\) concentration followed the standard protocol of the kit. To get data presented in Fig. 4B, Pg. \textit{A. actinomycetemcomitans} strains and Ss were grown for 48 hours, 24 hours and overnight respectively in TSB to early stationary phase under anaerobic conditions. Cells were resuspended in fresh CDM and 10% inoculated into fresh CDM to get bacterial suspensions. 50 µl of bacteria suspensions were mixed with 50 µl of Hydrogen Peroxide Assay solution and incubated under laboratory atmospheric conditions at 37 °C using the Synergy H1 Hybrid Reader. The optical density (OD\(600\)) for cell growth and fluorescent signal for \(\text{H}_2\text{O}_2\) concentration were monitored continuously by the reader. For testing \(\text{H}_2\text{O}_2\) concentration in the transwell system (96 Well PCR Support System transwell, Corning™ HTS Transwell\(^\text{TM}\)), Au and Ss cells were grown to early stationary phase in TSB medium, followed by resuspension in fresh CDM. 50 µl of bacteria suspension and 50 µl of Hydrogen Peroxide Assay solution were mixed at the bottom of the well. The insert was filled with 50 µl of CDM or 50 µl of \textit{Aa} suspension. After 30 minutes of reaction, the insert was discarded and the \(\text{H}_2\text{O}_2\) concentration at the bottom of the well was measured. Three replicates were performed to calculate the means and standard deviations.

**Statistical analysis.** All data were obtained from at least three biological replicates. Student’s t-test was applied to analyze data on biofilm biomass, \(\text{H}_2\text{O}_2\) concentration and CFU.

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Acknowledgements

We are extremely grateful to Dr. Marvin Whiteley (Georgia Institute of Technology) for providing the strains *A. actinomycetemcomitans* VT1169 and *A. actinomycetemcomitans* VT1169 ΔkatA, and to Dr. Donald Demuth (University of Louisville School of Dentistry) for providing the strain *A. actinomycetemcomitans* 652. CLSM was performed at Virginia Commonwealth University, Department of Anatomy & Neurobiology Microscopy Facility, supported, in part, by funding from NIH-NINDS Center Core Grant 5 P30 NS04763 and, in part, by funding from the NIH-NCI Cancer Center Support Grant P30 CA016059. This work was supported by National Institutes of Health grants R01DE023078 and R01DE018138 (PX). The funders had no role in study design, data collections and interpretation, or the decision to submit the work for publication.

Author Contributions

B.Z. and PX. conceived and designed this study, B.Z. carried out all of the experiments with the assistance of L.M. and E.N., B.Z. and PX. performed all of the experiments with the assistance of L.M. and E.N., B.Z. and PX. analyzed the data and wrote this manuscript. All authors reviewed and discussed the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-41467-9.

Competing Interests: The authors declare no competing interests.

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