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Selective killing of *Aggregatibacter actinomycetemcomitans* by ciprofloxacin during development of a dual species biofilm with *Streptococcus sanguinis*

Peter Suci [a,b,*], *Mark Young* [a,c]

[a] *Department of Plant Sciences, Montana State University, Bozeman 59717, MT, United States*
[b] *Center for Biofilm Engineering, Montana State University, Bozeman 59717, MT, United States*
[c] *Department of Chemistry & Biochemistry, Montana State University, Bozeman 59717, MT, United States*

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ABSTRACT

Objectives: Periodontal disease is associated with a pathogen-induced transition to a chronic destructive inflammatory response. Since commensals may either passively or actively contribute to immune homeostasis, therapies aimed at selectively reducing the competitive advantage of pathogens may be effective supplements to traditional methods. We developed an in vitro system to grow biofilms composed of the pathogen (*Aggregatibacter actinomycetemcomitans*) and the commensal (*Streptococcus sanguinis*). We used the biofilm model to determine the feasibility of selectively killing the pathogen using the fluoroquinolone, ciprofloxacin.

Design: Biofilms were exposed to relevant ciprofloxacin doses during the first 24 h of development, with subsequent removal of the ciprofloxacin for a 24 h period. Biofilm growth was assessed by confocal laser scanning microscopy, crystal violet staining and DNA abundance.

Results: Exposure to 0.01 mg/L or 0.5 mg/L ciprofloxacin significantly reduced the microcolony size and cell surface density of *A. actinomycetemcomitans* in the dual species biofilm over a 24 h period whilst allowing uninhibited *S. sanguinis* biofilm formation. *A. actinomycetemcomitans* biofilm development was insignificant over a subsequent 24 h period after removal of the ciprofloxacin indicating that *A. actinomycetemcomitans* cells were killed.

Conclusions: *A. actinomycetemcomitans* residing in a dual species biofilm with the commensal, *S. sanguinis* can be selectively killed, or at least rendered metabolically inactive, by treatment with ciprofloxacin. The dual species biofilm model will be a useful tool for designing in vivo studies to determine the efficacy of selective killing agents as an adjunct treatment of localized aggressive forms of periodontal disease.

* *Corresponding author at*: Department of Plant Sciences, Montana State University, Bozeman 59717, MT, United States. Tel.: +1 406 994 7219.
E-mail address: peter_s@erc.montana.edu (P. Suci).

Abbreviations: anti-Aa Ab, monoclonal antibody against *Aggregatibacter actinomycetemcomitans*; CLSM, confocal laser scanning microscope; CV, crystal violet.

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1. Introduction

Diseases associated with chronic inflammation are amongst the most difficult to treat.[1–3] In periodontal disease a cascade of events, triggered by changes in the oral microbial consortium, result in tissue and bone destruction that is orchestrated primarily by components of the immune system.[4–6] This chronic immune response is both destructive and unproductive since it does not eliminate the pathogens.[7]

The oral cavity is similar to the gut in that it is colonized by a complex consortium of commensals in healthy individuals.[8] Commensal microbes in the gut play an active role in passivating the inflammatory response to pathogens.[9] It is likely that oral commensals play a significant role in maintaining immune homeostasis in the oral cavity.[10] However, there is no direct evidence to support this claim.

Traditional methods of treatment of periodontal disease aim at removing or inactivating both pathogens and commensals indiscriminately.[11] In contrast, in a healthy state, the immune system's management of the oral consortium likely involves targeted inactivation of low levels of pathogens by a productive acute inflammatory response, since the oral mucosal immune system responds selectively to pathogens.[12,13] The implication is that selective killing of pathogens by antimicrobials could enhance or supplement normal immune function and mitigate the transition to a chronic inflammatory immune response. The key determining factor in restoring immune homeostasis by this strategy would be a reduction in the pathogen presence relative to that of the commensals, which might be achieved most effectively during the regrowth period after a conventional treatment.

Putative pathogens of periodontal disease are primarily gram-negative bacteria.[14–16] This suggests that the appropriate dose of a fluoroquinolone might selectively target periodontal pathogens in a biofilm consortium, since fluoroquinolones are generally more efficacious against gram-negative bacteria than gram-positive bacteria.[17,18] In particular the ciprofloxacin MICs for strains of the oral commensal *Streptococcus sanguinis* (0.5–16 mg/L) are substantially higher than the MICs for strains of *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) (0.002–0.006 mg/L),[19,20] the primary etiological agent of localized aggressive periodontitis.[21,22] A possible caveat to this approach that can be addressed initially with in vitro systems is that the oral microbial population is primarily in the biofilm form[16] and the biofilm mode of growth can affect the efficacy of antimicrobials in complex and indeterminate ways.[23] In the case of oral biofilms, there is an additional possible complicating factor of the contribution of consortium interspecies interactions to antimicrobial efficacy.

In vitro multispecies oral biofilm model systems have been developed previously to investigate interspecies interactions, as well as the influence of antimicrobials and nutrients on the biofilm composition.[24–27] Here, we developed an in vitro dual species biofilm model composed of *A. actinomycetemcomitans* and *S. sanguinis* to determine whether exposure to ciprofloxacin could be used to control the consortium composition. The biofilm model is a tool that can be used to aid in the rational design of in vivo experiments to test the hypothesis that selective killing of pathogens will enhance restoration of immune homeostasis.

2. Methods

2.1. Bacterial strains and media

A. actinomycetemcomitans strain D7S, a rough colony clinical isolate obtained from the central incisor of an African American female patient with generalized aggressive periodontitis was provided by Casey Chen, University of Southern California. *S. sanguinis* SK36, used previously for biofilm studies,[28] was obtained from ATCC (BAA-1455). Media consisted of modified tryptic soy broth (MTSB)[29] with 50 mL foetal bovine serum (HyClone) and 15 g bacto agar added (per litre) for solid medium, brain heart infusion broth (BHI) with 15 g bacto agar added (per litre) for solid medium, and a defined biofilm medium (BM) with 1% sucrose used previously to culture *S. sanguinis* biofilms.[28] Frozen stocks were maintained at −80 °C in 20% glycerol, 80% MTSB (*A. actinomycetemcomitans*) or BHI (*S. sanguinis*).

2.2. Biofilm culturing

All culturing was performed at 37 °C in 5% CO_2. Biofilms were cultured in 96 well polystyrene microtiter plates (Falcon Optilux™, Fisher Scientific) in BM with 10% MTSB added. This medium was chosen on the basis of preliminary studies which indicated that *A. actinomycetemcomitans* did not grow perceptibly in BM over a 48 h period. *S. sanguinis* biofilms did form in MTSB but the biofilms were so tenuously attached to the surface that a single rinse step would remove them. The *A. actinomycetemcomitans* biofilm inoculum was prepared by looping colonies, cultured on solid medium for 60 h, into a 1 mL aliquot of liquid medium. This suspension of cell aggregates was dispersed by repeated pipetting followed by forcing the suspension through a small bore needle (25G 7/8, Becton Dickinson). This suspension, which still consisted of cell aggregates with a range of sizes, was then filtered through two 5 μm pore filters (National Scientific Target Nylon Syringe Filter, 30 mm, Fisher Scientific) in tandem and diluted into the biofilm medium to obtain approximately 10^7 CFU/mL (based on an OD measurement). The *S. sanguinis* biofilm inoculum was prepared by inoculating 100 μL of a 60 h 5 mL BHI broth culture started from a frozen stock into 5 mL fresh BHI, incubating for 24 h, adjusting the cell density to approximately 10^8 CFU/mL (based on an OD measurement), and making a 1:1000 dilution of this cell suspension, either into biofilm medium (*S. sanguinis* single species biofilm inoculum) or into an aliquot of the *A. actinomycetemcomitans* biofilm inoculum (*A. actinomycetemcomitans*/*S. sanguinis* dual species biofilm inoculum), to obtain approximately 10^5 CFU/mL in both cases. The biofilms were inoculated by filling each well with 100 μL of the appropriate cell suspension (either pure *A. actinomycetemcomitans*, pure *S. sanguinis* or a mixture of *A. actinomycetemcomitans* and *S. sanguinis*) and incubating for 1 h. The inoculum was then replaced by fresh medium (200 μL) with or without ciprofloxacin. This medium was replaced at 24 h by fresh medium without ciprofloxacin (200 μL) for biofilms cultured for 48 h.

2.3. Antimicrobial treatment

Biofilms were treated with ciprofloxacin by filling wells with a solution of ciprofloxacin in the culture medium and incubating under the culturing conditions for 24 h. A 1 mg/mL ciprofloxacin solution was made from ciprofloxacin hydrochloride in methanol (ICN Biomedicals Inc. Catalogue # 199020) and subsequently diluted to 20 mg/L in sterile nanopure water. Appropriate volumes of this solution were added to culture medium to obtain 0.01 and 0.5 mg/L solutions. These solutions were filtered through a 0.2 μm filter before use.

2.4. Biofilm visualization

Confocal laser scanning microscope (CLSM) images were acquired with a Leica TCS-SP2-AOBS. Biofilm microcolonies were visualized through the floor of the wells by using a previous protocol.[29] Images were collected with a 40× 0.8 NA HCX APO L U-V-I water immersion objective. For CLSM visualization biofilms were labelled with an antibody specific for *A. actinomycetemcomitans* and a nucleic acid stain (SYTO 59, Invitrogen) that labels both *A. actinomycetemcomitans* and *S. sanguinis*. Monoclonal antibody (Aa-mAb) 225AA2[30] against *A. actinomycetemcomitans*, isotype mIgG2b, was donated by Rudolf Gmür, University of Zürich and purified as described previously.[29] The Aa-mAb 225AA2 epitope has been shown to be highly specific for *A. actinomycetemcomitans*.[30] No detectable cross reaction of the Aa-mAb with *S. sanguinis* planktonic cells was detected using a dot blot immunoassay, or with *S. sanguinis* biofilm using CLSM. For staining, biofilms were first washed with 1% BSA in 10 mM PBS (pH 7.0, 100 mM NaCl) for 5 min. Biofilms were subsequently rinsed with 0.85% saline between each step. Antibody and nucleic acid stain were also prepared in 0.85% saline. Biofilms were exposed to Aa-mAb at approximately 50 μg/mL for 50 min followed by exposure to a 1:100 dilution of fluorescently labelled secondary Ab (Alexa Fluor 488 rabbit antimouse IgG, Invitrogen) for 50 min, and finally a 15 μM SYTO 59 solution for 10 min. Green fluorescence from the anti-mouse secondary antibody and red fluorescence from the SYTO 59 nucleic acid stain were discriminated using Acousto-optical tunable filters.[29] Images were acquired at 2.0 μm intervals throughout the depth of the biofilms. Stacks were combined in Imaris software (Bitplane AG, Zürich, Switzerland) to yield final images. *A. actinomycetemcomitans* colony biovolumes were determined using the Imaris volumetric analysis software add-on.[26]

2.5. Crystal violet assay

Biofilms were rinsed with PBS and then exposed to a 1:4 dilution of the crystal violet (CV) solution (Protocol Crystal Violet, Fisher Scientific) for 15 min. This was followed by three rinses with nanopure water and addition of 200 μL of ethanol to each well. After a 1 h incubation period each well was mixed by pipeting and 1:10 dilutions were made in separate wells to allow for absorbances above 1. The absorbance of each well (including both diluted and undiluted wells) at 600 nm was then acquired using a plate reader. Values reported are estimates for undiluted wells containing 200 μL of the solution with the background from a sterile well exposed to the same CV solution and rinses subtracted.

2.6. Quantification of cell surface densities based on DNA abundance

DNA was extracted from biofilms by first lysing the cells in situ in the microtiter wells and then isolating the DNA using a GenElute™ Bacterial Genomic DNA kit (Sigma–Aldrich). Cells were lysed using a modification of a protocol designed for *S. sanguinis*.[31] Modifications were made to enhance lysis of the gram-negative *A. actinomycetemcomitans*. Wells were rinsed 3 times with 200 μL of 20 mM Tris, 5 mM EDTA, 100 mM NaCl, pH 7.0. Mutanolysin (Sigma–Aldrich, M9901) was added to obtain approximately 500 U/mL and the plate was incubated for 90 min at 37 °C. RNase (Sigma–Aldrich) was added to obtain a concentration of 0.5 mg/mL followed by addition of lysozyme (Fisher Scientific, BP535-1) to obtain a concentration of 2 mg/mL and the plate was incubated for 45 min at 37 °C. Proteinase K (Sigma–Aldrich, P2308) was added to obtain a concentration of 1 mg/mL and the plate was incubated for 45 min at 55 °C. N-lauryl sarcosine (Sarkosyl, Fisher Scientific) was added to obtain an 0.8% solution and pronase (Protease from *Streptomyces griseus*, Sigma–Aldrich, P5147) was added to obtain approximately 300 U/mL and the plate was incubated for 45 min at 55 °C. The contents of each well was removed and saved and 30 μL of 10% SDS was added to each well and the solution was mixed by pipeting. The contents of each well was added back and the plate was incubated for 6 h at 55 °C. Subsequent to this step the manufacturer's instructions were followed to purify the DNA from the lysate beginning with the addition of 200 μL of lysis solution C contained in the kit, vortexing for 15 s and incubation at 55 °C for 15 min. The final elution volume was 200 μL.

Quantitative PCR (qPCR) was used to quantify the DNA extracted from biofilms. Primers homologous to regions in the 16S RNA coding portion and specific for *A. actinomycetemcomitans* and *S. sanguinis* were designed with the NCBI primer designing tool (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were CTC AGG ACG AAC GCT GGC GG (forward, Ss), ATC CCC CGC TAC CAG GCA GG (reverse, Ss), CCG GCT AAC TCC GTG CCA GC (forward, Aa), AGG TCC GCC TAC GTG CCC TT (reverse, Aa). Primers were designed explicitly to not crosshybridize with the non-targeted species and this was confirmed using conventional PCR and agarose gel electrophoresis. Quantification was performed by using a Rotor-gene Q thermocycler (Qiagen) with a 15 min incubation period at 95 °C followed by 50 cycles of amplification. Each cycle consisted of 15 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C. The 25 μL reaction volumes contained 12.5 μL SYBR Green ER qPCR SuperMix (Invitrogen), 5.5 μL molecular biology grade water (Sigma–Aldrich, W4502), 1 μL of 5 pmol/μL forward and reverse primers and 5 μL of the sample. Threshold times determined from the fluorescence data curves were converted to DNA copy number based on standard curves generated from purified genomic DNA, quantified spectrophotometrically using a NanoDrop ND-100 spectrophotometer (Thermo Scientific). DNA copy number was converted to biofilm cell density (cells/cm^2) by multiplying by 40 to account for the 200 μL elution volume, dividing by the number of 16S copies in

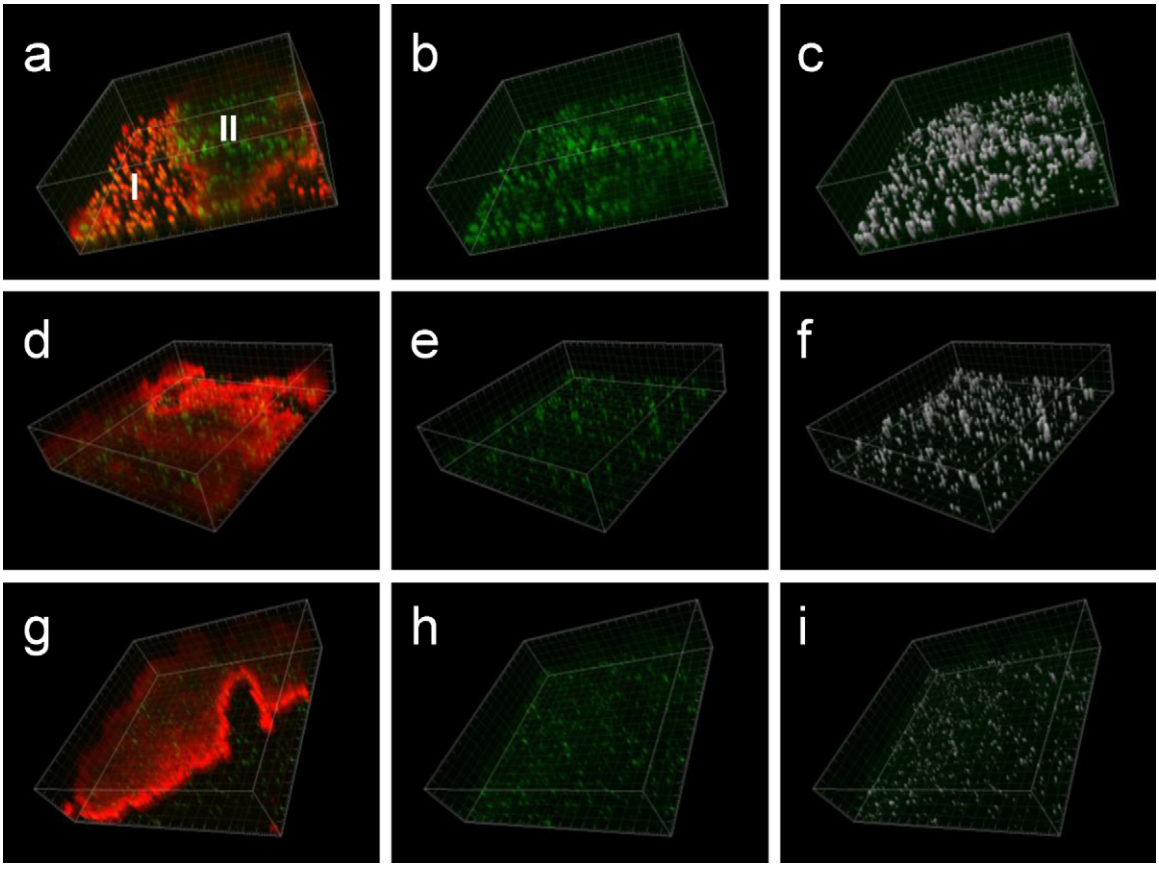

Fig. 1 – CLSM images of dual species biofilms. (a–c) No ciprofloxacin treatment; (d–f) 0.01 mg/L ciprofloxacin; (g–i) 0.5 mg/L ciprofloxacin. (a, d and g) (left column) Composites of the red and green fluorescence channels. (b, e and h) (middle column) Green channel (*A. actinomycetemcomitans* microcolonies). (c, f and i) (right column) Surface representations of the green channel used to compute *A. actinomycetemcomitans* microcolony volumes. Tick marks are at 4 μm spacings yielding a field size of 380 μm × 380 μm. Regions labelled I and II in (a) are discussed in the text.

the *A. actinomycetemcomitans* or *S. sanguinis* genome (6 and 4, respectively), and diving by 0.196 to account for the well area (0.196 cm^2).

2.7. Statistical analysis

Statistical significance of differences in means was evaluated by an unpaired *t* test for three replicates of the experimental conditions being compared.

3. Results

3.1. Selective killing visualized by confocal microscopy

In dual species biofilms composed of the oral pathogen, *A. actinomycetemcomitans* and the oral commensal, *S. sanguinis*, *A. actinomycetemcomitans* formed discrete microcolonies on the surface that were overlaid in some portions with a lawn of *S. sanguinis*. Fig. 1 presents images of 48 h biofilms. Exposure to ciprofloxacin during the first 24 h of co-species biofilm development selectively inhibited the growth of *A. actinomycetemcomitans* biofilm as indicated by the reduced colony sizes in treated biofilms (Fig. 1d–i) compared to untreated biofilm (Fig. 1a–c). Both species were stained with a red nucleic acid stain whilst *A. actinomycetemcomitans* was labelled with an *A. actinomycetemcomitans*-specific antibody coupled to a fluorescent secondary antibody, rendering the *A. actinomycetemcomitans* cells green. Regions of partial *S. sanguinis* biofilm coverage revealing the edge of the *S. sanguinis* lawn are shown. Images in the left column are composites of the red and green fluorescence channels whilst images in the middle column consist of only the green channel (*A. actinomycetemcomitans* microcolonies). In the right column are the surface representations of the green channel used to compute *A. actinomycetemcomitans* microcolony biovolumes. Biovolumes computed from these surface representations are 37.2, 8.22 and 3.01 in units of 10^4 μm^3 for Fig. 1a, d and g, respectively. (Data for replicates are presented below). Untreated *A. actinomycetemcomitans* microcolonies appear green in the composite image in the region of Fig. 1a indicated by II, whereas colonies in the region not colonized with *S. sanguinis* (region I) appear red. Coverage by the *S. sanguinis* biofilm in region II was apparent when the field was observed through the ocular using epifluorescence, even though the red fluorescence in the confocal image is very faint. We propose that the green hue of the *A. actinomycetemcomitans* microcolonies in region II of Fig. 1a is due to an optical effect originating from attenuation of the red

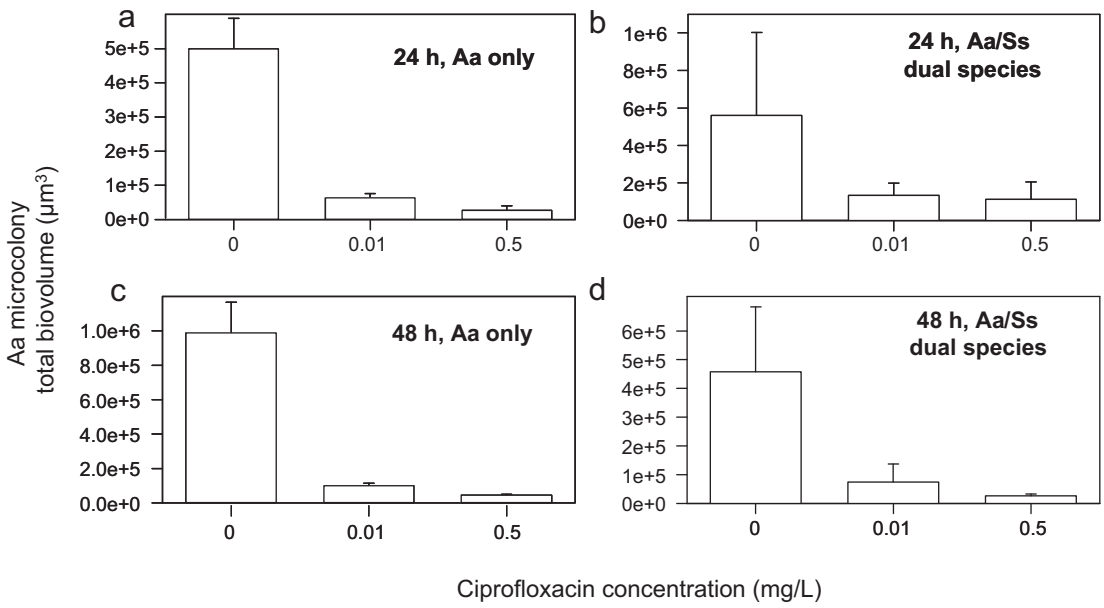

Fig. 2 – *A. actinomycetemcomitans* microcolony biovolumes showing inhibition of *A. actinomycetemcomitans* over a 24 h period, and killing evidenced by lack of growth during a subsequent 24 h period after removal of ciprofloxacin. (a) Single species 24 h *A. actinomycetemcomitans* (Aa) biofilm; (b) Dual species 24 h *A. actinomycetemcomitans/S. sanguinis* (Aa/Ss) biofilm (c) Single species 48 h *A. actinomycetemcomitans* (Aa) biofilm (d) Dual species 48 h *A. actinomycetemcomitans/S. sanguinis* (Aa/Ss) biofilm. Data are total biovolume over the field of view (380 μm × 380 μm). Error bars are standard deviations.

fluorescent signal by the relatively thick *S. sanguinis* biofilm. We observed that the treated *A. actinomycetemcomitans* microcolonies stained more faintly with the nucleic acid stain than the untreated colonies in single species biofilms (data not shown). We propose that this phenomenon allowed the green fluorescence from antibody-labelled *A. actinomycetemcomitans* microcolonies to dominate in the composite images of treated biofilms even in regions where they were not overlaid with a lawn of *S. sanguinis* biofilm (Fig. 1d and g, left column).

3.2. Selective killing assessed by biovolume and biomass

A. actinomycetemcomitans microcolony biovolumes computed from CLSM images show that exposure to 0.01 or 0.5 μm/mL ciprofloxacin for 24 h inhibited *A. actinomycetemcomitans* biofilm formation during development of both single and dual species biofilms (Fig. 2a and b). *A. actinomycetemcomitans* microcolonies in both single and dual species biofilms exhibited no increase in size during a subsequent 24 h period after removal of the ciprofloxacin indicating that *A. actinomycetemcomitans* cells were inactivated (Fig. 2c and d). Reported biovolumes are the means of the total *A. actinomycetemcomitans* microcolony volume per field of view. *A. actinomycetemcomitans* microcolony biovolumes for untreated 24 h and 48 h biofilms are all significantly different from those of treated biofilms at the 0.01 level of significance for all conditions (for both single and dual species biofilms). Compared to 24 h biofilms, *A. actinomycetemcomitans* microcolony size in dual species biofilms was actually reduced by 45 and 70% in 48 h biofilms treated with 0.01 and 0.5 mg/L ciprofloxacin, respectively. For the 0.5 mg/L treatment this reduction was significant at the 0.05 level (p value, 0.0216).

The CV assay is typically used as an index of biofilm biomass.[28,32] CV absorbance values indicate that *S. sanguinis* biofilm formation was uninhibited by exposure to ciprofloxacin in the dual species biofilms (Fig. 3). *A. actinomycetemcomitans* biofilm microcolonies in the treated dual species biofilms are relatively small compared to the *S. sanguinis* lawn which is expected to dominate the biomass. The contribution of *A. actinomycetemcomitans* biofilm microcolonies to the biomass in dual species biofilms was quantified by using the CV absorbance to biovolume ratio in untreated *A. actinomycetemcomitans* biofilm as a conversion factor. Using this factor to convert *A. actinomycetemcomitans* biovolumes, which were directly measured in single species biofilms (Fig. 2), to estimates of the percent contribution of the *A. actinomycetemcomitans* biofilm to the CV absorbance in the dual species biofilms yields 1.5 and 0.5% for biofilms treated with 0.5 and 0.01 mg/L ciprofloxacin, respectively. Accounting for this small contribution of *A. actinomycetemcomitans* biofilm to the biomass of dual species biofilms, differences between mean biomass of the untreated single species *S. sanguinis* biofilm and *S. sanguinis* biomass in both the treated dual species biofilms are insignificant (p values >0.1), both for the 24 h and 48 h time periods. In contrast to treated biofilms, *A. actinomycetemcomitans* biofilm made a significant contribution (23.2%) to the biomass of the untreated dual species biofilm. Taking this contribution into account, the biomass of the *S. sanguinis* biofilm for the untreated condition was approximately 60% less in the dual species biofilm compared to the single species biofilm. This difference is significant at the 0.01 level. There is approximately a 20% reduction in *S. sanguinis* biomass in the 48 h treated single species biofilms compared to the 48 h untreated biofilm which are significant at the 0.01 level.

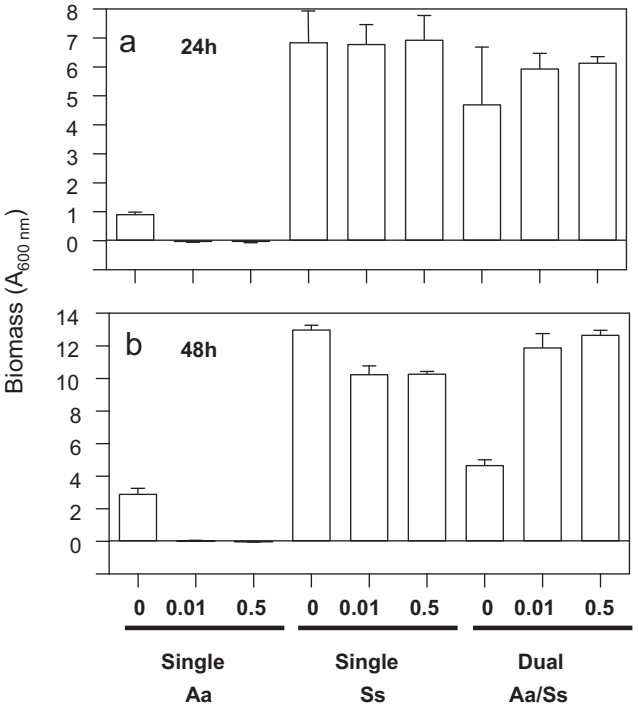

Fig. 3 – Quantification of biomass using CV indicating that *S. sanguinis* (Ss) biofilm development is uninhibited by ciprofloxacin at either the 0.01 or the 0.5 mg/L dose in the dual species biofilms. (a) 24 h biofilms; (b) 48 h biofilms. The graphs show the extent of CV staining ($A_{600\ nm}$) versus various conditions. The contribution of *A. actinomycetemcomitans* (Aa) to the biomass in the dual species *A. actinomycetemcomitans/S. sanguinis* (Aa/Ss) treated biofilms is less than 2% as discussed in the text. The lower biomass of the untreated dual species biofilm may be due to the influence of the underlying *A. actinomycetemcomitans* microcolonies on *S. sanguinis* biofilm formation or detachment. Absorbances were below background for three of the treated single species biofilms yielding negative values for the background subtracted data. Numbers on the abscissa refer to ciprofloxacin concentrations as in Fig. 2. Error bars are standard deviations.

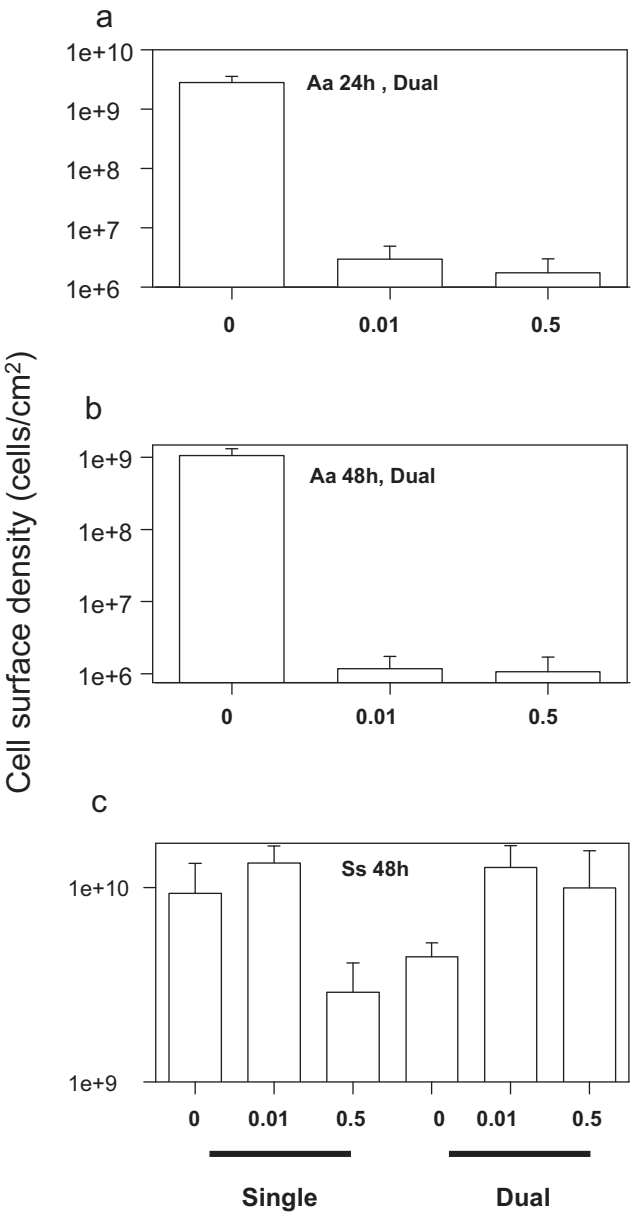

Fig. 4 – Cell surface densities assessed by qPCR showing selective killing of *A. actinomycetemcomitans* (Aa) in the dual species biofilm. (a) *A. actinomycetemcomitans* (Aa) cell surface densities in the 24 h dual species biofilm; (b) *A. actinomycetemcomitans* (Aa) cell surface densities in the 48 h dual species biofilm; (c) *S. sanguinis* (Ss) cell surface densities in single and dual species 48 h biofilms. Numbers on the abscissa refer to ciprofloxacin concentrations as in Fig. 2. Error bars are standard errors.

3.3. *Selective killing assessed by DNA content*

According to qPCR data, exposure to ciprofloxacin for 24 h at both 0.01 and 0.5 mg/L reduced cell surface density of *A. actinomycetemcomitans* in the dual species biofilm by more than two orders of magnitude compared to the untreated control (Fig. 4a). There was no increase in *A. actinomycetemcomitans* biofilm cell surface density during the 24 h period following removal of the ciprofloxacin indicating that *A. actinomycetemcomitans* biofilm had been inactivated (Fig. 4b). Differences in *A. actinomycetemcomitans* cell surface density for treated biofilm and biofilm treated with 0.01 and 0.5 mg/L ciprofloxacin are significant at the 0.05 level for both 24 h and 48 h biofilms. Similar to the biovolume data, there was actually a decrease in cell surface density over the 24 h period following removal of the ciprofloxacin of 60 and 39%, for dual species biofilms treated with 0.01 and 0.5 mg/L ciprofloxacin, respectively; however, these reductions are not significant at the 0.05 level. *S. sanguinis* cell surface densities are between 2.8×10^9 and 1.3×10^{10} cells/cm^2 in single and dual species biofilms, both treated and untreated with ciprofloxacin (Fig. 4c). Consistent with the biovolume data, the *S. sanguinis* cell surface density was lower for the untreated condition in the

dual species biofilm. However, this reduction is not significant at the 0.05 level. The *S. sanguinis* cell surface density was also lower for the single species biofilm treated with 0.5 mg/L, but means are not significantly different at the 0.05 level. Assuming that more replicates would increase the level of significance of this result, this is consistent with the biovolume data and may reflect an effect of the higher ciprofloxacin dose (0.5 μm/mL) on the *S. sanguinis* biofilm which is mitigated in the dual species biofilm.

4. Discussion

In order to test the hypothesis that oral commensals contribute to reestablishment of immune homeostasis during treatment of periodontal disease methods that can be used in vivo to selectively eliminate pathogens are needed. The possibilities range from relatively sophisticated targeting approaches[29,33,34] to general application of conventional antimicrobials which are known to have some selectivity.[35] The complexity of possible factors influencing antimicrobial action against biofilms demands that the feasibility of proposed strategies be verified on in vitro biofilm models before moving to animal models or clinical trials. The dual species biofilm we developed could be exploited to probe the efficacy of both targeted and more generally selective (non-targeted) strategies.

Our results suggest that exposure of periodontal surfaces to ciprofloxacin could prevent establishment of a mature *A. actinomycetemcomitans* biofilm during a regrowth period following conventional treatment whilst allowing unhindered biofilm formation by the commensal, *S. sanguinis*. Periodontitis is treated initially by mechanical removal of plaque, with local or systemic application of antibiotics reserved for more recalcitrant cases.[36] We followed a ciprofloxacin dosing regime that can be attained by systemic application.[37,38] *A. actinomycetemcomitans* resistance to ciprofloxacin is rare, or perhaps non-existent.[39] The lack of inhibition of *S. sanguinis* by the higher (0.5 mg/L) ciprofloxacin dose leaves an allowance margin for possible development of *A. actinomycetemcomitans* resistance. Although fluoroquinolones have been used to treat periodontitis,[40,41] there has been no systematic study conducted to test whether selective elimination occurs in vivo and whether it improves the clinical outcome. Such studies may reveal that more sophisticated highly selective targeting approaches are essential to prevent detrimental shifts in the commensal population composition, or prevent selection of resistant pathogens.

Our strategy for selective killing was predicated on differential planktonic MICs for the pathogen and the commensal. One implication is that the greater efficacy of fluoroquinolones against gram-negative bacteria compared to gram-positive bacteria might be leveraged to selectively reduce the pathogen load in other forms of periodontitis. However, it cannot be assumed a priory that this relatively simple strategy will have general application since the biofilm mode of growth can profoundly alter the efficacy of antimicrobials, most commonly by conferring an intrinsic resistance. The key factor here may be that biofilms were exposed to the antimicrobial during the regrowth period. Most theories to explain biofilm resistance revolve around qualities that manifest as the biofilm matures.[16,23,42]

We anticipated that interspecies interactions would play a more obvious role in the dual species biofilm development. *A. actinomycetemcomitans* is able to utilise a metabolic byproduct of Streptococcal fermentation as a nutrient and hydrogen peroxide produced by Streptococci at subinhibitory levels is sensed by *A. actinomycetemcomitans*.[27] Conversely, it was shown that *A. actinomycetemcomitans* produces a compound that is toxic to Streptococci.[43] There are, in fact, hints of possible cospecies interactions in our data. *S. sanguinis* biofilm was less abundant in untreated dual species biofilms suggesting that *A. actinomycetemcomitans* biofilm might inhibit *S. sanguinis* biofilm formation or induce detachment. *A. actinomycetemcomitans* microcolony size was reduced in untreated dual species biofilms compared to untreated single species *A. actinomycetemcomitans* biofilms. The inhibition of *A. actinomycetemcomitans* by Streptococcal hydrogen peroxide could be responsible for this. However, a more obvious explanation would be nutrient limitation in the static system. The next logical step in pursuing in vitro studies is to use a flowing system which has the advantage of being more like the in vivo situation but with the disadvantage of being less high through-put.

The reduction in *A. actinomycetemcomitans* microcolony biovolume for treated versus untreated biofilm is less pronounced than the reduction in cell surface densities (Figs. 2 and 4). This could be a consequence of artefacts in either of the measurements. However, in support of the quality of the data, the correspondence between biovolumes and cell surface densities for untreated biofilm are reasonable. Specifically, the volume per cell based on the ratio of biovolume to cell surface density is approximately 0.35 μm^3, equivalent to a cube having dimensions of 0.7 μm per side. *A. actinomycetemcomitans* cells are relatively small rods with dimensions less than 1 μm[44] and cells are fairly tightly packed in the biofilm microcolonies.[45] Exposure of a susceptible strain of *Escherichia coli* (MIC 0.012 mg/L) to 0.012 mg/L ciprofloxacin for 40 min induces DNA fragmentation followed by diffusion of the fragments outside the cell after a relatively mild lysis step.[46] It seems possible that *A. actinomycetemcomitans* cell walls would become sufficiently permeable to allow diffusion of DNA fragments through the cell wall during a 24 h exposure period. This would be consistent with the reduced nucleic acid staining of treated *A. actinomycetemcomitans* microcolonies noted in Section 3, and would explain the discrepancy in relative reductions in biovolume and cell surface densities of the treated biofilms compared to untreated biofilms.

In summary, we have developed a relevant dual species biofilm model composed of a periodontal pathogen and an oral commensal and presented measurement techniques that enable its characterization. In addition, we demonstrated a relatively simple means of controlling the composition of the dual species consortium. From a general perspective, methods to manipulate biofilm consortia in situ provide tools for investigating the hypothesis that oral commensals play a critical role in maintenance of healthy immune function. The significance of testing this hypothesis lies not only in finding better approaches for clinical treatment of periodontal disease, but in understanding immune interactions with complex biofilm consortia.[22]

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REFERENCES

1. Southerland JH, Taylor GW, Moss K, Beck JD, Offenbacher S. Commonality in chronic inflammatory diseases: periodontitis, diabetes, and coronary artery disease. *Periodontology* 2000, 2006;**40**:130–43.
2. Barnes PJ, Larin M. Mechanisms of disease – Nuclear factor κB – A pivotal transcription factor in chronic inflammatory diseases. *New England Journal of Medicine* 1997;**336**:1066–71.
3. Aloisi F, Pujol-Borrell R. Lymphoid neogenesis in chronic inflammatory diseases. *Nature Reviews Immunology* 2006;**6**:205–17.
4. Marton IJ, Kiss C. Protective and destructive immune reactions in apical periodontitis. *Oral Microbiology and Immunology* 2000;**15**:139–50.
5. Garlet GP, Cardoso CRB, Campanelli AP, Ferreira BR, Avila-Campos MJ, Cunha FQ, et al. The dual role of p55 tumor necrosis factor-α receptor in *Actinobacillus actinomycetemcomitans*-induced experimental periodontitis: host protection and tissue destruction. *Clinical and Experimental Immunology* 2007;**147**:128–38.
6. Taubman MA, Valverde P, Han XZ, Kawai T. Immune response: the key to bone resorption in periodontal disease. *Journal of Periodontology* 2005;**76**:2033–41.
7. Trombone APF, Ferreira SB, Raimundo FM, de Moura KCR, Avila-Campos MJ, Silva JS, et al. Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. *Journal of Periodontal Research* 2009;**44**:443–51.
8. Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunology Letters* 2004;**93**:97–108.
9. Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, et al. Prokaryotic regulation of epithelial responses by inhibition of I κB-alpha ubiquitination. *Science* 2000;**289**:1560–3.
10. Roberts FA, Darveau RP. Beneficial bacteria of the periodontium. *Periodontology* 2000, 2002;**30**: 40–50.
11. Mombelli A, Samaranayake LP. Topical and systemic antibiotics in the management of periodontal diseases. *International Dental Journal* 2004;**54**:3–14.
12. Chino T, Santer DM, Giordano D, Chen C, Li C, Chen CH, et al. Effects of oral commensal and pathogenic bacteria on human dendritic cells. *Oral Microbiology and Immunology* 2009;**24**:96–103.
13. Beklen A, Sorsa T, Konttinen YT. Toll-like receptors 2 and 5 in human gingival epithelial cells co-operate with T-cell cytokine interleukin-17. *Oral Microbiology and Immunology* 2009;**24**:38–42.
14. Listgarten MA. Pathogenesis of periodontitis. *Journal of Clinical Periodontology* 1986;**13**:418–25.
15. Tanner A, Kent R, Maiden MFJ, Taubman MA. Clinical, microbiological and immunological profile of healthy, gingivitis and putative active periodontal subjects. *Journal of Periodontal Research* 1996;**31**:195–204.
16. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontology* 2000, 2002;**28**:12–55.
17. Avery RK. Adding gram-positive prophylaxis to fluoroquinolone regimens reduces streptococcal bacteraemias, but prophylaxis remains controversial. *Cancer Treatment Reviews* 2004;**30**:385–8.
18. Appelbaum PC, Hunter PA. The fluoroquinolone antibacterials: past, present and future perspectives. *International Journal of Antimicrobial Agents* 2000;**16**:5–15.
19. Hoogkamp-Korstanje JAA, Roelofs-Willemse J. Comparative in vitro activity of moxifloxacin against gram-positive clinical isolates. *Journal of Antimicrobial Chemotherapy* 2000;**45**:31–9.
20. Muller HP, Holderrieth S, Burkhardt U, Hoffler U. In vitro antimicrobial susceptibility of oral strains of *Actinobacillus actinomycetemcomitans* to seven antibiotics. *Journal of Clinical Periodontology* 2002;**29**:736–42.
21. Fine DH, Kaplan JB, Kachlany SC, Schreiner HC. How we got attached to *Actinobacillus actinomycetemcomitans*: a model for infectious diseases. *Periodontology* 2000, 2006;**42**:114–57.
22. Henderson B, Nair SP, Ward JM, Wilson M. Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. *Annual Review of Microbiology* 2003;**57**:29–55.
23. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001;**358**:135–8.
24. Kuboniwa M, Tribble GD, James CE, Kilic AO, Tao L, Herzberg MC, et al. *Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Molecular Microbiology* 2006;**60**:121–39.
25. Filoche SK, Zhu M, Wu CD. In situ biofilm formation by multi-species oral bacteria under flowing and anaerobic conditions. *Journal of Dental Research* 2004;**83**:802–6.
26. Periasamy S, Kolenbrander PE. *Aggregatibacter actinomycetemcomitans* builds mutualistic biofilm communities with *Fusobacterium nucleatum* and *Veillonella* species in saliva. *Infection and Immunity* 2009;**77**:3542–51.
27. Ramsey MM, Whiteley M. Polymicrobial interactions stimulate resistance to host innate immunity through metabolite perception. *Proceedings of the National Academy of Sciences of the United States of America* 2009;**106**:1578–83.
28. Ge XC, Kitten T, Chen ZM, Lee SP, Munro CL, Xu P. Identification of *Streptococcus sanguinis* genes required for biofilm formation and examination of their role in endocarditis virulence. *Infection and Immunity* 2008;**76**:2551–9.
29. Suci P, Kang S, Gmur R, Douglas T, Young M. Targeted delivery of a photosensitizer to *Aggregatibacter actinomycetemcomitans* biofilm. *Antimicrobial Agents and Chemotherapy* 2010;**54**:2489–96.
30. Gmür R, Thurnheer T. Monoclonal antibodies for the rapid identification in clinical samples of *Peptostreptococcus micros* and *Actinobacillus actinomycetemcomitans* serotypes, a, d, and e. *Medical Microbiology Letters* 1996;**5**:335–49.
31. Ganeshkumar N, Song M, McBride BC. Cloning of a *Streptococcus sanguis* adhesin which mediates binding to saliva-coated hydroxyapatite. *Infection and Immunity* 1988;**56**:1150–7.
32. Flemming K, Klingenberg C, Cavanagh JP, Sletteng M, Stensen W, Svendsen JS, et al. High in vitro antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms. *Journal of Antimicrobial Chemotherapy* 2009;**63**:136–45.
33. Robinson AM, Creeth JE, Jones MN. The use of immunoliposomes for specific delivery of antimicrobial agents to oral bacteria immobilized on polystyrene. *Journal of Biomaterials Science-Polymer Edition* 2000;**11**:1381–93.
34. Embleton ML, Nair SP, Heywood W, Menon DC, Cookson BD, Wilson M. Development of a novel targeting system for lethal photo sensitization of antibiotic-resistant strains of

Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* 2005;**49**:3690–6.
35. Beikler T, Prior K, Ehmke B, Flemmig TF. Specific antibiotics in the treatment of periodontitis-a proposed strategy. *Journal of Periodontology* 2004;**75**:169–75.
36. Greenstein G. Nonsurgical periodontal therapy in 2000: a literature review. *Journal of the American Dental Association* 2000;**131**:1580–92.
37. Conway TB, Beck FM, Walters JD. Gingival fluid ciprofloxacin levels at healthy and inflamed human periodontal sites. *Journal of Periodontology* 2000;**71**:1448–52.
38. Tozum TF, Yildirim A, Caglayan F, Dincel A, Bozkurt A. Serum and gingival crevicular fluid levels of ciprofloxacin in patients with periodontitis. *Journal of the American Dental Association* 2004;**135**:1728–32.
39. Madinier IM, Fosse TB, Hitzig C, Charbit Y, Hannoun LR. Resistance profile survey of 50 periodontal strains of *Actinobacillus actinomycetemcomitans*. *Journal of Periodontology* 1999;**70**:888–92.
40. Guentsch A, Jentsch H, Pfister W, Hoffmann T, Eico S. Moxifloxacin as an adjunctive antibiotic in the treatment of severe chronic periodontitis. *Journal of Periodontology* 2008;**79**:1894–903.
41. Kleinfelder JW, Mueller RF, Lange DE. Fluoroquinolones in the treatment of *Actinobacillus actinomycetemcomitans* associated periodontitis. *Journal of Periodontology* 2000;**71**:202–8.
42. Takahashi N, Ishihara K, Kato T, Okuda K. Susceptibility of *Actinobacillus actinomycetemcomitans* to six antibiotics decreases as biofilm matures. *Journal of Antimicrobial Chemotherapy* 2007;**59**:59–65.
43. Hammond BF, Lillard SE, Stevens RH. A Bacteriocin of *Actinobacillus actinomycetemcomitans*. *Infection and Immunity* 1987;**55**:686–91.
44. Rhodes ER, Shoemaker CJ, Menke SM, Edelmann RE, Actis LA. Evaluation of different iron sources and their influence in biofilm formation by the dental pathogen *Actinobacillus actinomycetemcomitans*. *Journal of Medical Microbiology* 2007;**56**:119–28.
45. Kaplan JB, Meyenhofer MF, Fine DH. Biofilm growth and detachment of *Actinobacillus actinomycetemcomitans*. *Journal of Bacteriology* 2003;**185**:1399–404.
46. Tamayo M, Santiso R, Gosalvez J, Bou G, Fernandez JL. Rapid assessment of the effect of ciprofloxacin on chromosomal DNA from *Escherichia coli* using an in situ DNA fragmentation assay. *BMC Microbiology* 2009:9.