Author's response to reviews

Title: Development of an in vitro periodontal biofilm model for assessing antimicrobial and host modulatory effects of bioactive molecules

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Author's response to reviews: see over
Reviewers comments: 1

1. I suggest using error bars on all SEM images for clarity.

_We thank the reviewer for pointing this out and all SEM images have had scale bars added._

2. To me, it would make sense to combine Fig. 4A with 4D, 4B with 4E and 4C with 4F. At present the data from the controls are duplicated.

_The graphs have been changed accordingly. Figures 4A and D are now Figure 4A, Figures 4B and E are now Figure 4B and Figures 4C and 4F are now Figure 4C._

Minor Essential Revisions:

1. The key controls for the qPCR reactions need to be clearly stated in the Methods or Results. Were primer sets tested against each organism in the model to ensure that they were specific? Were products analysed by agarose gel electrophoresis and/or sequencing to ensure that they were correct? Were melting curves performed? All essential data for the qPCR experiment should be given, as listed in the MIQE guidelines (see http://www.rdml.org/miqe.php).

_We apologise for the lack of clarity here. We had already included the following section, but have added to this to make clearer. Although we did not state undertaking agarose gel electrophoresis, this was performed, but we did not feel it appropriate to add due to the extensive methodology already present. We have included the references from which the primers were taken. Given that these are published then the requirement for further information is not relevant. We apologise for not including this in the first manuscript._

_Samples were quantified to calculate the colony forming equivalent (CFE) based upon a previously established standard curve of bacterial colony forming units ranging from $1 \times 10^3$ to $10^8$ cfu/mL. The $R^2$ values for these standard curves ranged from 0.956 to 0.994. Melting curve analysis was performed for all primer sets to ensure a single peak, which was indicative of primer specificity._

2. Methods are also needed for the Alamar Blue assay.

_Alamarblue assay was performed following manufacturer’s instructions. The company from which it was purchased and its methods have been updated as follows:_

>*Multispecies biofilms were incubated in the co-culture model for 4 and 24 h and the viability of the epithelial cells ± active treatments (CHX and RSV) evaluated using a metabolic assay of 10% v/v alamarBlue®, according to the manufacturer’s instructions (Life Technologies, Paisley, UK). After 4 h incubation the absorbance was read at 570 nm and the reference wavelength*
“at 600 nm, and the percentage reduction in biofilm viability calculated using the manufacturer’s formula.”

3. The extracellular matrix is not clear in Fig. 2B or 2C (line 262). Arrows might help to highlight the matrix, but I am not sure that I can see anything clear in any case. Of course, SEM is not a good method for visualising the matrix since it will collapse during dehydration. The ECM is also mentioned in line 396.

As mentioned by the reviewer SEM is not a good method for visualising matrix, as such we have removed both references to this in the text.

4. Why was S. mitis standardized to a higher OD than other bacteria? (line 132)

Bacteria were all standardized to an O.D., which corresponds to $1 \times 10^8$ CFU/ml. The structure of the sentence may have been confusing. As such the sentence has been reworded as follows.

The bacteria were washed with PBS then standardized to an OD$_{550}$ of 0.2, except for S. mitis, which was standardized to an OD$_{550}$ of 0.5, in a colorimeter to obtain approximately $1 \times 10^8$ cfu/mL of each bacterial species on their specified day of use.

5. Were denture based samples really used (line 182)? There is no mention of them anywhere else as far as I can see.

Reference to denture based samples was an unintentional mistake and has been removed. The sentence now reads as follows.

The fixed and dried samples were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope [14].

6. There are a number of typographical errors that need correcting:

a. line 28: only one ‘development’ is needed here.

   Altered as follows:

   Understanding how different oral hygiene products influence inflammatory properties is important for the development of new products.

b. line 50: down-regulated.

   Altered as follows:

   RSV-treated epithelial cells in co-culture were down-regulated in the release of IL-8 protein, but not mRNA.
c. line 370: ‘Additionally...’ should be a full sentence.

*Altered as follows:*

Additionally, increases in gene expression of a variety of pro-inflammatory chemokines and cytokines including IL-6, IL-8, TNF, CSF-2, CXCL1 and CXCL3 at 4 h compared with the cells only control were observed.

d. line 399: an immediate bactericidal (not bacteriostatic)...  

*Altered as follows:*

This data agrees with previous work of the mechanisms of action of CHX which show an immediate bacteriocidal action combined with a prolonged bacteriostatic action due to absorption of the active to the surface.

e. line 413: ‘of the skin’ – neither of these bacteria are really skin colonisers. P. aeruginosa is an aquatic species that is an opportunistic pathogen primarily in cystic fibrosis or in wounds and E. coli is mainly found in the gut.

*Altered as follows:*

RSV has been reported to have antimicrobial effects both against planktonic and bacterial biofilms, with most studies focusing on bacteria such as P. aeruginosa and E. coli with MIC ranging from 5-50µg/ml [24, 25].

f. Figure 4A and 4D y-axis label should just read ‘fold change relative to...’.

*Graphs have been changed accordingly.*

Reviewers comments 2:

- Major Compulsory Revisions

1. lane 263.
   It is hard to assert that S. mitis is dominant from the SEM data.

   *We are willing to concede that it may be difficult to observe the S. mitis species and that the more obvious morphotype is F. nucleatum, thus we have amended the sentence as such:*

   The biofilm was shown to be a dense complex of different morphotypes dominated by F. nucleatum.

2. Fig. 4.
   Control data seem to be dually used in Fig. 4. Combine data of Fig. 4A and 4D.
Combine data of Fig. 4B and 4E.
Combine data of Fig. 4C and 4F.

The graphs have been changed accordingly. Figures 4A and D are now Figure 4 A, Figures 4B and E are now Figure 4B and Figures 4C and 4F are now Figure 4C.

3. Some figures and the legends.

The captions in some figures should be written more reader-friendly. It is difficult to understand some figures mean at a glance. For example, in Fig. 4B, the treatment labels “Biofilms”, “CHX”, and “Media” on the X-axis should be replaced by “Untreated biofilms”, CHX-treated biofilms, and “Vehicle control”, respectively.

Figure legends have been changed in all figure 4 graphs. “Biofilms” has now been replaced with ‘Untreated Biofilms’, “RSV” has now been replaced with “RSV treated cells”, “CHX” has been replaced with “CHX treated biofilms” and “Media” has now been replaced with “Vehicle control”.

4. Discussion section.

Characterization of biofilm molecules responsible for the regulation of IL-8 expression is ideally desired. Otherwise, at least discuss about the relationships between IL-8 expression and specific oral bacteria or the components, with referring representative reports that described about them. Many readers might be interested in the possible mechanisms in which what is responsible for the regulation of IL-8.

We have included the following sentence: ‘Recently, it has been shown that biofilms including the ‘red complex’ organisms such as P. gingivalis or F. nucleatum increased IL-8 production during early host interaction, yet with longer exposure the chemotactic factors are downregulated, presumably through the release of proteolytic gingipains’

5. l. 56 and l.421.

IL-8 expression was only examined in this study. Are other chemokines/cytokines or signaling pathway molecules enhanced by the biofilms formed in the models. Authors conclude that it did appear to suppress epithelial cells from releasing inflammatory mediators. The arguments must be strengthened by additional analysis about other candidate molecules as well.

This sentence was already present in the discussion. We have added an additional sentences.
“Additionally, increases in gene expression of a variety of pro-inflammatory chemokines and cytokines including IL-6, IL-8, TNF, CSF-2, CXCL1 and CXCL3 at 4 h compared with the cells only control were observed.”

“These dynamic changes in pro-inflammatory mediators demonstrate that there is interplay between the complex biofilm and the epithelial cells.”

However, many of the other pro-inflammatory molecules did tend to decrease at 4 h, such as IL-6 and IL-1β.

though CXCL5, the neutrophil activating peptide was decreased transcriptionally, which may be involved in reducing neutrophil activation [32].

6. Fig. 2B, 2C, 3E-J
Show the scale for each fig.

Scale bars have been added to SEM images

7. Fig. 3E, 3H, and 3J.
Replace these figs with still higher magnification photos.

The highest magnification is currently x5000 and we believe there is nothing to be gained from further magnified images. However we have moved the images to a separate page to allow increased size of the images to allow better viewing.