Measuring *Streptococcus mutans, Streptococcus sanguinis and Candida albicans* biofilm formation using a real-time impedance-based system

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Abstract

Candida albicans and streptococci are amongst the most common fungal and bacterial organisms present in the oral cavity, with a growing body of evidence implicating *C. albicans* in increased caries severity and in the formation of the cariogenic biofilm. However, the interactive mechanisms between cariogenic streptococci and *Candida* are yet to be elucidated. In this study, the real-time biofilm formation of *C. albicans*, *S. mutans* and *S. sanguinis* was assessed individually and in combination using the xCELLigence system, an impedance-based microbial biofilm monitoring system. The impedance signal was the highest for *C. albicans*, followed by *S. mutans* and *S. sanguinis*. Although the streptococcal mixed adhesion was found to follow a similar trend to that of *S. sanguinis*, the introduction of *C. albicans* resulted in higher adhesion patterns, with the combined growth of *S. sanguinis* and *C. albicans* and the combination of all three species resulting in higher biofilm formation than any of the individual organisms over time. This study, the first to use impedance for real-time monitoring of interkingdom biofilms, adds to the body of evidence that *C. albicans* and oral streptococcal adhesion are interlinked and suggests that interkingdom interactions induce changes in the oral biofilm dynamics over time.

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1. Introduction

A previous study describing the prevalence of caries in a population of pregnant women, also reported on an increase in oral candidiasis amongst these women (Africa and Turton, 2019). Candidiasis during pregnancy may be favoured by the oral acidic environment created by hormonal changes, increased sugar consumption and gastric acid exposure (during recurrent morning sickness) (Hey-hadavi, 2002; Annan and Nuamah, 2005), bringing about a change in the normal microbiota of the mouth, favouring the growth of *Candida* (Rio et al., 2017) and the formation of dental caries (Vasiliauskiene, 2003; Kidd, 2005; Silk et al., 2008; Rakchanok et al., 2010). Caries activity has largely been associated with *Streptococcus mutans* and *S. sobrinus*, with recent studies reporting similar virulence factors expressed by *Candida albicans* (Mayer et al., 2013; Pereira et al., 2018).

With *Candida albicans* and streptococci being amongst the most common fungal and bacterial organisms present in the oral cavity (Marsh and Zaura, 2017), there is a growing body of evidence implicating *C. albicans* in the formation of the cariogenic biofilm (Cavalcanti et al., 2015; Hirota et al., 2017). Recent studies revealed high salivary *Candida* carriage associated with caries severity in both adults (De-la-Torre et al., 2016) and children (Lozano Moraga et al., 2017), thereby recognising *Candida* as an indicator of caries activity (Raja et al., 2010) and possible vertical transmission from mothers to early caries affected children (Berkowitz, 2003; Weintraub et al., 2010; Xiao et al., 2016). The precise mutualistic and synergistic mechanisms of the interaction between *Candida* and the oral streptococci implicated in caries have yet to be determined (Falsetta et al., 2014; Ellepola et al., 2017; Fernandes et al., 2018; Koo et al., 2018). To our knowledge, the application of impedance microbiology (using the xCELLigence analyser) to support the synergy between *Streptococcus mutans* and *Candida albicans* in the cariogenic biofilm has not previously been reported.

The xCELLigence real time cell analyser (RTCA), employs microtiter plates with gold microelectrodes through which impedance signals (expressed as cell index, CI) are measured and allows for real-time monitoring of changes in cell number, size, adhesion and EPS biofilm formation, quantitative assessment of cell adhesion and continuous and automated data analysis, thereby having an advantage over traditional biofilm staining and quantification assays (van Duuren et al., 2017). The arrangement of microelectrodes at the bottom of the wells, as used in this system, has been found to better match biofilm environmental characteristics (Paredes et al., 2014). This technology has been used to assess the biofilm formation abilities of individual bacterial species (Junka et al., 2012; van Duuren et al., 2017) and was found to be suitable for both *Candida* and oral streptococci, with the results complementing conventional biofilm methods (Alshanta et al., 2019; Gutiérrez et al., 2016).

The objective of the present study was to investigate the interspecies and interkingdom biofilm formation of the oral commensal organisms *C. albicans*, *S. mutans* and *S. sanguinis* by assessing their adhesion / extracellular polymeric substances (EPS) formation dynamics over time.

2. Materials and Methods

2.1 Organisms used in this study

Type strains were obtained from the American Type Culture Collections (ATCC, Manassas, VA) and included *Candida albicans* (ATCC 90028), *Streptococcus mutans* (ATCC 25175) and *Streptococcus sanguinis* (ATCC 10556). The freeze-dried *C. albicans* isolate was revived by

growth in 10 ml Sabouraud dextrose broth (Cat. no. CMO147, Oxoid, UK) while the *S. mutans* and *S. sanguinis* were revived in 10 ml brain heart infusion (BHI) broth (Cat. no. CM1135, Oxoid, UK), followed by incubation at 37°C for 3–5 days. Purity of growth for *C. albicans* was confirmed by streaking the broth culture onto Sabouraud dextrose agar (Cat. no. 84088, Sigma-Aldrich, USA) and incubating aerobically at 37°C for 24–48 h, while the two streptococci were streaked on blood agar plates, followed by anaerobic incubation at 37°C for 24–48 h.

2.2 Preliminary toxicity testing

In order to assess whether any of the organisms induced toxicity against the others, toxicity tests were performed. The organisms were aseptically transferred to 15 ml Greiner tubes containing 8 ml yeast peptone dextrose broth (Cat. no. Y1375, Sigma-Aldrich, USA) (*C. albicans*) and brain heart infusion broth (Cat. no. CM1135, Oxoid, UK) (*S. mutans* and *S. sanguinis*), followed by incubation at 37°C for 24 h. The tubes were subsequently centrifuged at 3000 *g* for 10 minutes and the supernatant was collected using a sterile Pasteur pipette. The supernatant was dispensed into a sterile 50 ml Greiner tube, aspirated using a sterile syringe and filtered using a 0.2 µm syringe filter into a sterile 15 ml Greiner tube. Assurance of supernatant sterility was performed by inoculating the filtered suspension in a Sabouraud dextrose (*C. albicans* supernatant) or blood agar plate (*S. mutans* and *S. sanguinis* supernatants) and incubating at 37°C for 24 h aerobically and anaerobically, respectively, with no growth being observed.

A 0.5 McFarland standard suspension (100 µl) of each organism was placed in the wells of a microtiter plate containing growth media (BHI broth) when testing the two streptococci and tryptone yeast extract broth (3 g/L yeast extract (Cat. no. 70161, Sigma-Aldrich, USA) and 5 g/L casein hydrolysate (Cat. no. 22090, Sigma-Aldrich, USA), pH 7.0) ultra-filtered using a Centrisart 10 kDa molecular-weight ultrafiltration unit (Cat. no. 13239E, Sigma-Aldrich, USA) and supplemented with 1% sucrose (UFTYE+1%S) when testing the streptococci and *C. albicans*), followed by the addition of 100µl of the sterile supernatant of the other two organisms to the wells. Experiments were performed in duplicate. The absorbance of the microtiter plates containing the individual microorganisms and sterile supernatants was read at 512 nm using an Anthos 2010 spectrophotometer (Cat. no. GF1755011, Biochrom, UK), followed by incubation at 37°C and subsequent readings at 8, 24, 36 and 48-hours.

2.3 Real time monitoring of biofilm formation

The microbial concentration changes of *C. albicans* were initially tested on yeast peptone dextrose (YPD) broth (Cat. no.Y1375, Sigma-Aldrich, USA), with 100 µl of a 1:20 dilution of a 0.5 McFarland standard suspension and 100 µl of YPD broth added to the wells of an E-plate 16 (Cat. no. 05469830001, ACEA Biosciences, USA). *Streptococcus mutans* and *S. sanguinis* were tested on tryptone soy broth (TSB) (Cat. no. 22092, Sigma-Aldrich, USA) with 1% sucrose (TSB+1%S), as this medium allows for biofilm formation in oral streptococci (Ge et al., 2008). One hundred microliters (100 µl) of a 1:10 dilution of a 0.5 McFarland standard suspension of each organism and 100 µl of TSB+1%S were placed in the wells of an E-Plate 16. Fifty microliters (50 µl) of each organism were added to wells with mixed growth, resulting in a 100 µl mixed cell suspension and 100 µl TSB+1%S. Sterile distilled water was placed in the surrounding evaporation-control troughs, as recommended by the manufacturer. The plate was then inserted into the RTCA plate analyser (Cat. no. 05469759001, ACEA Biosciences, USA), which was previously placed in a 37°C incubator. An experimental procedure was logged on the RTCA software package (Cat. no. 05454433001, ACEA Biosciences, USA), with impedance readings set to take place at 15-minute intervals for 48 hours.

For the interkingdom biofilms UFTYE+1%S broth was used, which allows for the growth of both genera of organisms (Ellepola et al., 2017; Falsetta et al., 2014). The dilution of the streptococci was 1:10 of a 0.5 McFarland standard suspension, while for *C. albicans*, which has a faster growth and adhesion rate, a 1:50 dilution of a 0.5 McFarland standard suspension was used. When assessing the adhesion changes of the three organisms growing together, a 33 µl suspension of each organism was added to the wells, resulting in a 100 µl mixed cell suspension and 100 µl UFTYE+1%S broth. Impedance readings were set to take place at 15-minute intervals for 48 hours. The real-time cell index values for the full duration of the experiments were then plotted in individual graphs. Experiments were performed in duplicate and sterility controls were included in every tested plate.

3. Results

After an adjustment period of 5 hours, the concentration of *C. albicans* on YPD was seen to increase exponentially up to 15 hours, after which it slowly decreased but continued having positive CI values (Figure 1).

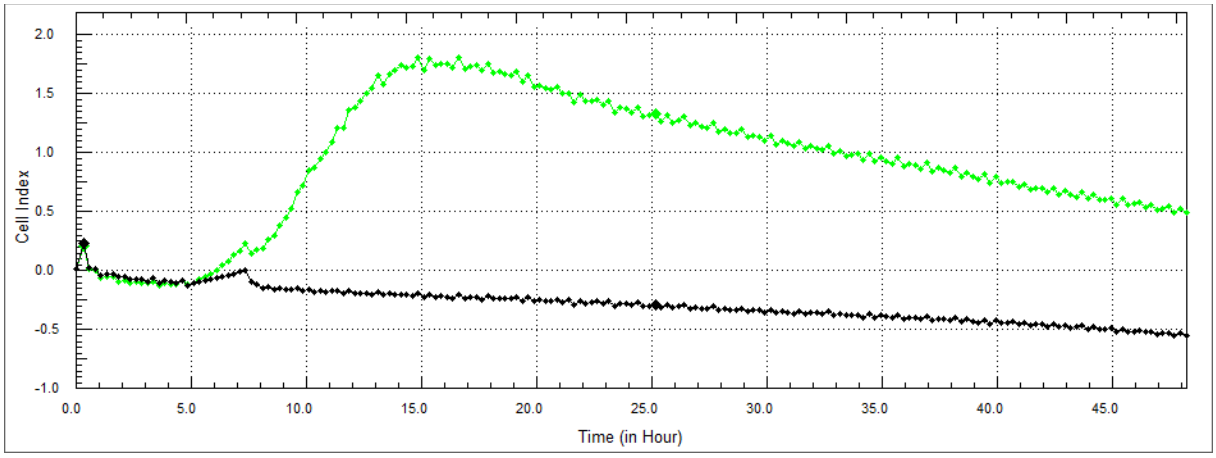


Figure 1. Cell index variations of *C. albicans* ATCC90028 (light green) biofilm formation on YPD broth, with the sterility control (only YPD broth) shown in black.

In the case of the streptococci grown on TSB+1%S, a similar pattern was observed for *S. mutans*, which showed a much higher biofilm/EPS formation than *S. sanguinis* (Figure 2). The adhesion of *S. sanguinis*, however, was seen to take place before that of *S. mutans* (with the maximum adhesion levels being reached at 9 hours and 16 hours respectively) and to reduce over time, with the mixed growth biofilm following a similar trend to that of *S. sanguinis* (Figure 2). The adhesion results on TSB+1%S clearly showed that in the presence of *S. sanguinis*, *S. mutans* adhesion is decreased. When performing the toxicity testing it was noted that *S. mutans* grew at a slower rate when in the presence of the *S. sanguinis* supernatant. There was no toxicity-associated growth reduction observed with the other streptococcal and *C. albicans* combinations of organisms and supernatants.

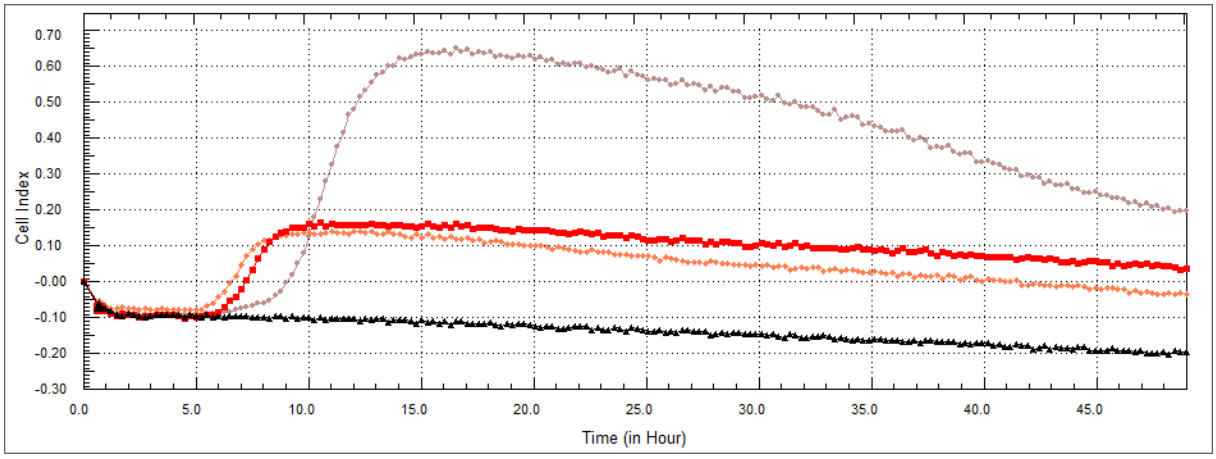
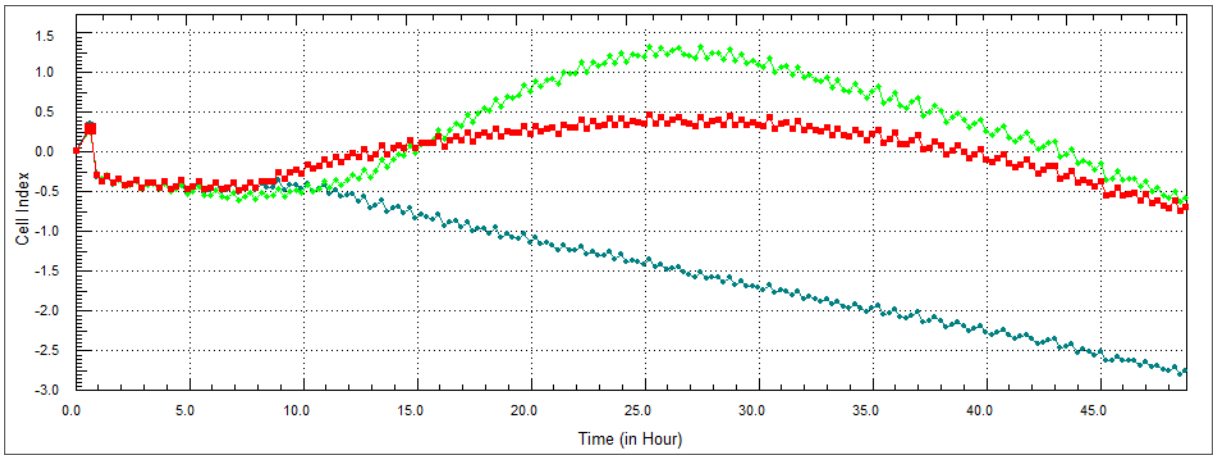
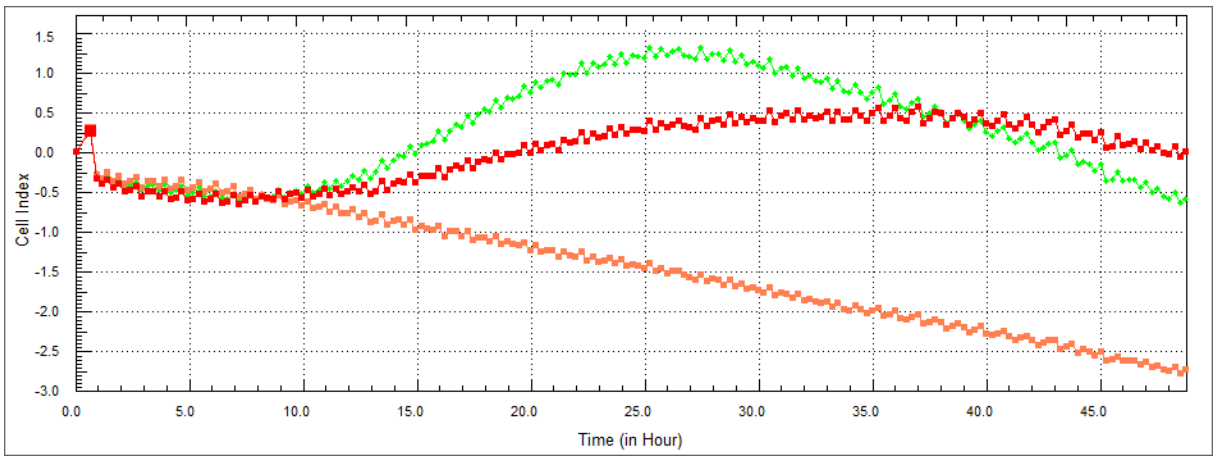


Figure 2. Cell index variations of *S. mutans* ATCC25175 (light grey), *S. sanguinis* ATCC10556 (coral) and mixed growth (red) biofilm formation on TSB+1%S, with the sterility control (only TSB+1%S) shown in black.

When growing the *C. albicans* and the streptococci on UFTYE+1%S, their adhesion patterns differed to those on YPD and TSB+1%S, respectively. While *C. albicans* formed biofilms on this medium, *S. mutans* and *S. sanguinis* did not adhere to the well surface/form EPS. However, when the organisms were combined, the adhesion dynamics changed considerably (Figure 3): in the case of *C. albicans* and *S. mutans*, the mixed growth resulted in higher CI readings between 9 hours and 14 hours, after which the values remained below those of *C. albicans* (3a). In the case of *C. albicans* and *S. sanguinis* mixed growth, the adhesion started off in between the curves for the individual species but surpassed that of *C. albicans* after 40 hours of incubation (3b). A very similar pattern was seen when all three species were combined (3c).



a



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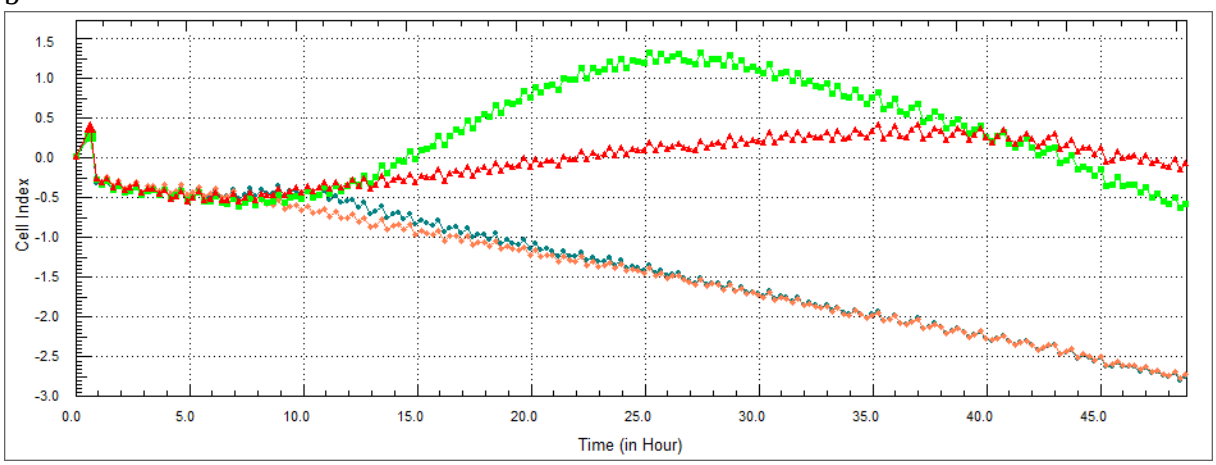

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Figure 3. (a) Cell index variations of *S. mutans* ATCC25175 (cyan), *C. albicans* ATCC90028 (light green) and mixed growth (red) biofilm formation on UFTYE+1%S broth. **(b)** Cell index variations of *S. sanguinis* ATCC10556 (coral), *C. albicans* ATCC90028 (light green) and mixed growth (red) biofilm formation on UFTYE+1%S broth. **(c)** Cell index variations of *S. mutans* ATCC25175 (cyan), *S. sanguinis* ATCC10556 (coral), *C. albicans* ATCC90028 (light green) and mixed growth (red) biofilm formation on UFTYE+1%S broth.

4. Discussion

An adequate understanding of the microbial interactions in the cariogenic biofilm is essential to developing caries prevention and control strategies. This study reports on *S. sanguinis*, one of the primary colonisers of the tooth surface, *S. mutans* which is strongly associated with the aetiology of dental caries and *C. albicans* which has recently been implicated in caries formation.

In order to obtain more detailed data, we opted to adjust the parameters to read the impedance signal at shorter time intervals and over a longer period of time than the usual 24 h reported in similar studies (Alshanta et al., 2019; Gutiérrez et al., 2016). We also chose not to change the culture media during the experiment, as this is known to affect the accuracy of the monitoring parameters (Kim et al., 2011). The steady drop of impedance values over time has been observed in similar monoculture studies on oral streptococci (Gutiérrez et al., 2016) and *C. albicans* (Alshanta et al., 2019). It is known that the CI values increase as the biofilm adheres and proliferates on the bottom of the E-plate wells, with a peak CI being consistent with the start of the biofilm maturation stage and a decrease taking place when the biofilm initiates the detachment phase (Gutiérrez et al., 2016). A declining slope after the peak CI value is reached has been suggested as a measure of biofilm formation (van Duuren et al., 2017). As the polysaccharides that hold the biofilm together are broken down by enzymes, releasing the cells from the biofilm matrix, the electrical signals are also further affected by extracellular components and physical appendages of microorganisms, which is consistent with the death phase (Garrett et al., 2008).

Our results of the mixed streptococcal biofilm agree with previous studies on the mixed growth of these two species: the competition between *S. mutans* and *S. sanguinis* has been documented, with *S. sanguinis* being associated with oral health (Belda-Ferre et al., 2012) and causing a delay in the colonisation of *S. mutans* (Caufield et al., 2000) by repressing its growth through H_2O_2 production (Kreth et al., 2005; Valdebenito et al., 2018), thereby reducing dental caries (Zhu et al., 2018). The H_2O_2 produced by *S. sanguinis* would also explain the slower growth of *S. mutans* in the presence of the *S. sanguinis* supernatant, which was observed when the toxicity testing between these organisms was conducted.

Although *C. albicans* adhered well when grown in the two tested media, the two streptococci grew but did not adhere well in the UFTYE+1%S media. *Streptococcus sanguinis* is known to vary in its biofilm formation according to the type of media used (Ge et al., 2008) and looking at the CI curves in the two media the same appears to be true for *S. mutans*. Although the two bacterial type strains did not adhere well on their own in UFTYE+1%S, their mixed growth biofilm with *C. albicans* over two days resulted in interesting observations, notably the increased biofilm formation between *S. mutans* and *C. albicans* in the adhesion and proliferation phase (but not subsequently) and the higher biofilm maturation and subsequent detachment after 40 hours seen between *S. sanguinis* and *C. albicans* and when the three species were grown together. These latter observations might have occurred since, as a primary coloniser, *S. sanguinis* might facilitate the attachment of other organisms (Zhu et al., 2018).

Although *C. albicans* interacts with *S. sanguinis* through cell wall proteins on both organisms (Xu et al., 2014), it does not interact strongly, nor does it directly bind to *S. mutans* (Koo et al., 2018). Interactions between these two species is only possible through the production of

glucosyltransferases by *S. mutans* and in the presence of high levels of sucrose (Koo et al., 2018). Being aciduric and acidogenic (Pereira et al., 2018), *C. albicans* may be responsible for promoting bacterial colonisation and biofilm formation with the primary colonizer *S. sanguinis* (Diaz et al., 2012), along with the induction of virulence genes and bacterial-yeast aggregation with *S. mutans* (Falsetta et al., 2014), thereby enhancing the formation of the *S. mutans* cariogenic biofilm (Sampaio et al., 2019).

An advantage of impedance microbiology is that it can easily be automated with results obtained within hours compared with the conventional plate count method which is more labour intensive. Limitations of the xCELLigence system include its inability to discriminate between cell adhesion and EPS formation. However, by showing the quantitative adhesion changes in mixed oral biofilm constituents over time and the shifts in biofilm formation stages when different organisms are grown together, it demonstrated that interkingdom interactions can be more accurately monitored using this technology. Further studies including electron microscopy imaging of biofilm formation at predetermined intervals as well as gene expression, will further inform and contribute to the knowledge base on the antagonism between *S. mutans* and *S. sanguinis*, the synergistic interaction between *C. albicans* and *S. mutans* within the cariogenic biofilm as well as the interactions between *C. albicans* and *S. sanguinis* within the non-cariogenic biofilm.

Authors' contributions

CA conceptualized and coordinated the study. PA conducted the laboratory investigations and prepared the first draft of the manuscript. Both authors were involved in the writing of the manuscript, gave their final approval and agree to be accountable for all aspects of the work.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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