Insights into the bacterial and fungal ecology of endodontic infections
Persoon, I.F.

Citation for published version (APA):
Persoon, I. F. (2016). Insights into the bacterial and fungal ecology of endodontic infections

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Toll-like receptor response to dual-species biofilms with *Candida albicans* and *Enterococcus faecalis*: a pilot in vitro study

Ilona F. Persoon  
Wim Crielaard  
Marcello P. Riggio  
Gordon Ramage  
David F. Lappin

Submitted

Clyde Arc in Glasgow, Scotland.

The Clyde Arc bridge crosses the river Clyde and has an innovative curved design. Similarly, a connection between bacteria and fungi can affect the host response.
ABSTRACT

AIM

*Candida albicans* and *Enterococcus faecalis* are associated with dental root canal infections in previously treated roots, causing an inflammatory response at the root apex. We hypothesise that we can determine the relative ability of these root canal pathogens to stimulate the inflammatory response and the efficacy of root canal irrigants with an *in vitro* model system.

METHODOLOGY

Immune cells were stimulated with planktonic or mono- or dual-species biofilms, either untreated or treated with the irrigants sodium hypochlorite (NaOCl) or chlorhexidine. Immune stimulation was assessed in an *in vitro* THP-1 monocyte model, where upon Toll-like receptor (TLR) stimulation and activation of nuclear factor kappa β and AP-1 pathways the enzyme secreted embryonic alkaline phosphatase (SEAP) is released. Monocyte activation was also determined by assessing TNF-α levels in culture fluids using an enzyme-linked immunosorbent assay.

RESULTS

Only untreated *E. faecalis* (planktonic *P* < 0.001, biofilms *P* < 0.001) and NaOCl-treated planktonic *E. faecalis* (*P* < 0.001) elicited significant TLR stimulation. *C. albicans* failed to stimulate TLRs. Furthermore, *E. faecalis* biofilm effects on TLR activation were nullified when the bacteria were treated and when co-cultured with *C. albicans*. In all cases, SEAP levels were significantly correlated to TNF-α levels.

CONCLUSION

Traditional root canal irrigants adequately reduced the effects of the infection on inflammatory cells *in vitro*. The interplay between the two microorganisms and the host resulted in decreased TLR stimulation which may lead to a modification of the innate immune response. This might have ramifications for therapy and be a subject worthy of further investigation.
INTRODUCTION

When microorganisms infect the previously sterile root canal system, this will cause the host to generate an inflammatory response at the tooth apex, namely apical periodontitis (Kakehashi et al. 1965). The host detects and responds to microorganisms making use of their evolutionary conserved components, which are located mostly on the surface of the microbe or liberated into the fluid phase (pathogen-associated molecular patterns; PAMPs). The inflammatory response to microbial infection is initiated when PAMPs bind to pattern recognition receptors (PRRs) on cell surfaces, of which Toll-like receptors (TLRs) are an important example. These activate several intracellular signalling pathways, of which the nuclear factor kappa β (NF-κβ) pathway is a major route (Bowie & O’Neill 2000). Microorganisms can activate TLRs in pulp tissue within the tooth (Staquet et al. 2008) and in granulomas at the root apex (Desai et al. 2011). Stimulation of inflammatory cells via these receptors leads to the production of reactive oxygen species and nitric oxide, causing inflammatory reactions and bone resorption at the apical site (Marcato et al. 2008).

Many microbial species are implicated in primary infections, but *Enterococcus faecalis* and *Candida albicans* are associated with apical periodontitis at previously treated teeth. They have been isolated from such root canal infections, both separately and together (Peciuliene et al. 2001). Post-treatment apical periodontitis occurs in approximately 36% of cases (Pak et al. 2012). *E. faecalis* is a Gram-positive gastrointestinal bacterium and produces aggregation substance, surface adhesin, cytolysin, bacteriocin, gelatinase (Sedgley et al. 2005), and extracellular superoxide (Huycke et al. 2002). The bacterium can survive antibiotic treatment (Sedgley et al. 2004), a wide temperature and pH range, hyperosmosis, and nutrient deprivation (Hartke et al. 1998). *C. albicans* is a dimorphic fungus, occurring in yeast and hyphal forms. The hyphal phenotype is especially associated with invasion of tissues and consequential mortality (Felk et al. 2002). When co-culturing *E. faecalis* and *C. albicans*, they clearly respond to each other’s presence and are able to form a biofilm together (Cruz et al. 2013; Fox et al. 2014). A biofilm is composed of microorganisms of one or more species which are encapsulated in a polysaccharide matrix adhered to a surface (Hall-Stoodley et al. 2004). The biofilm offers protection against a hostile host environment and allows for activation, development and increased complexity of microbial functions. Thus, microorganisms in biofilms are more virulent towards the host and more resilient to treatment.

Treatment of apical periodontitis aims at removing the microorganisms from the root canal system. Mechanical treatment is regularly supplemented with irrigation using disinfectants such as sodium hypochlorite (NaOCl) or chlorhexidine (CHX). In brief, NaOCl is a highly active oxidizing agent that reacts with proteins,
fatty acids and lipids resulting in membrane disruption and impaired cellular metabolism (McDonnell & Russell 1999), whereas the cationic CHX binds to the negatively charged phospholipids of the cell wall and cell membranes leading to membrane disruption and congelation of the cytoplasm (McDonnell & Russell 1999). Both these irrigants have antimicrobial activity against biofilms of *E. faecalis* and *C. albicans* (Valera et al. 2013).

Greater insight into the mechanisms involved in activating the immune system may clarify the pathogenesis of apical periodontitis and influence the selection of treatment modalities to dampen the potentially devastating side effects of the immune response to root canal infection. The aim of the present study was to determine the inflammatory response to apical periodontitis pathogens by measuring the ability of the microorganisms to stimulate an *in vitro* TLR assay model system – before and after the use of root canal irrigants. For this purpose we investigated the effect of *E. faecalis* and *C. albicans* singly and in combination on the activation of host immunity via TLRs and secretion of TNF-α.

**METHODOLOGY**

*Culture of microorganisms*

*Fusobacterium nucleatum* (DSMZ, Braunschweig, Germany) was used as a positive control for TLR stimulation (Marcato et al. 2008). It was grown on Fastidious Anaerobic Agar (Oxoid, Basingstoke, UK) for 48 hours under anaerobic conditions (H₂ and N₂) at 37°C. *F. nucleatum* was then inoculated in Schaedlers Anaerobic Broth (Oxoid) and grown for 48 hours under anaerobic conditions at 37°C. *E. faecalis* E2, which was isolated from a root canal infection, was selected for use in this study (Sedgley et al. 2004). The commensal *Streptococcus oralis* (DSMZ) was used as a negative control (Tanner et al. 1998). *E. faecalis* and *S. oralis* were grown on Tryptic Soy Agar (TSA; Oxoid, Basingstoke UK) overnight at 5% CO₂ in air at 37°C. Both microorganisms were inoculated in Tryptic Soy Broth (TSB; Sigma-Aldrich, Poole, UK) overnight at 5% CO₂ in air at 37°C. The positive control *Listeria monocytogenes* was supplied heat-killed by InvivoGen (Toulouse, France). *C. albicans* SC5314 was inoculated from a freezer stock on Sabouraud agar (Sigma-Aldrich) overnight aerobically at 37°C, after which it was inoculated in Yeast Peptone Broth (YPD; Sigma-Aldrich) overnight, shaking aerobically at 37°C.

*Preparation and treatment of planktonic microorganisms*

Overnight cultures were washed twice in phosphate-buffered saline (PBS; Sigma-Aldrich) and diluted in PBS to obtain stock solutions at a density of 10⁹ bacteria per ml or 10⁸ fungal cells per ml. Suspensions were diluted in PBS and into culture
medium to obtain the different multiplicities of infection (MOI) at 1 to 10 yeast and 1 to 100 bacteria per monocyte. Total microbial inactivation through root canal irrigation was mimicked by incubating *E. faecalis* or *C. albicans* for 1 hour in either 2% (w/v) NaOCl (Sigma-Aldrich), 2% CHX (GSK, Weybridge, UK) or PBS. All cells were then washed twice in PBS, except for the NaOCl-treated cells. These were washed once in 5% (w/v) sodium thiosulphate (Sigma-Aldrich) and once in PBS. Total microbial inactivation was ascertained by overnight culturing of *E. faecalis* on TSA and *C. albicans* on Sabouraud agar.

**Preparation and treatment of microbial biofilms**

Overnight cultures were washed twice in PBS and diluted in artificial saliva (Pratten *et al.* 1998) to a density of 10⁷ enterococci per ml and 10⁶ *Candida* cells per ml. Artificial saliva contained porcine stomach mucins (0.25% w/v; Sigma-Aldrich), sodium chloride (0.35% w/v; VWR, Lutterworth, UK), potassium chloride (0.02% w/v; Sigma-Aldrich), calcium chloride dihydrate (0.02% w/v; VWR), yeast extract (0.2% w/v; Oxoid), lab lemco powder (0.1% w/v; Oxoid), proteose peptone (0.5% w/v; Oxoid), and urea (0.05% v/v; Oxoid) in ddH₂O. Biofilms were cultivated on 13 mm diameter Thermanox coverslips (Fisher Scientific, Loughborough, UK) within Costar 24-well plates. Biofilms were grown for 24 hours at 37°C under aerobic conditions, after which the biofilm cover slips were treated with the irrigants and washed with PBS as described above. Total microbial inactivation was ascertained by a cell viability assay using alamar blue (Thermo Fisher). Biofilm formation was ascertained visually and by phase contrast light microscopy.

**Cell culture**

THP1-XBlue cells and THP1-XBlue-defMyD cells (InvivoGen) derived from the human monocytic THP-1 cell line. They were cultured in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with penicillin, streptomycin, 200 µg/mL Zeocin (and 200 µg/mL Hygromycin for MyD88 deficient cells only), and 10% fetal calf serum in a humidified atmosphere with 5% CO₂ in air at 37°C.

**NF-κβ /AP-1-reporter assay**

THP1-XBlue cells express a NF-κβ and alkaline phosphatase (AP)-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. The substrate QUANTI-Blue is converted by SEAP and its levels are determined by a colour change using a spectrophotometer at 630 nm. THP1-XBlue-defMyD cells are deficient in the MyD88 adaptor protein and therefore do not respond to activation of receptors that are dependent on MyD88 for second signalling, such as TLRs.
For the assay with planktonic microorganisms, THP1-XBlue cells were seeded into a 96-well microtitre plate at $2 \times 10^5$ cells per well in RPMI-1640 with 10% fetal calf serum and incubated in a humidified atmosphere with 5% $CO_2$ in air at 37°C. For every microorganism, the optimal MOI was determined by titration, i.e., the lowest MOI that gave a peak response in the SEAP assay. This was determined to be 100:1 for *E. faecalis* and 10:1 for *C. albicans* per cell. These organisms were added singly or together to the THP-1 pro-monocyte cultures. PBS was used as a negative control, while heat-killed *L. monocytogenes* at a density of 100 bacteria per THP-1 monocyte served as a positive control. The experiment was performed in duplicate on at least three occasions. These experiments were repeated with THP1-XBlue-defMyD cells.

To assess TLR activation by the microbial biofilms, cells were seeded into a 24-well microtitre plate at $1 \times 10^6$ cells per well in RPMI-1640 with 10% fetal calf serum and incubated in a humidified atmosphere with 5% $CO_2$ in air at 37°C. The coverslips with the biofilms were mounted inverted upon Costar Transwell cell culture inserts (Fisher Scientific) and put over the wells with the THP-1 cells, leaving them 0.5 mm from the bottom of the well adjacent to the cells, as previously described (Millhouse *et al.* 2014). Cover slips exposed to artificial saliva for 24 hours served as a negative control. Supernatant was extracted after 21 hours. The experiment was performed in duplicate on three occasions.

The cell supernatant was added in a 1:10 ratio (20 µL to 180 µL) to QUANTI-Blue in a 96-well microtitre plate. The plate was incubated at 37°C for 4 hours, after which SEAP levels were determined using a MRX II microplate reader (Dynex technologies, Chantilly, VA, USA) at 630 nm.

**Quantitative polymerase chain reaction**

An approximation of the microbial and extracellular DNA present in the biofilm was determined to quantify levels of microorganisms within the biofilms. Genomic DNA was prepared from the biofilms and from known quantities of *E. faecalis* and *C. albicans* (colony forming unit equivalents; CFUeq.). The standard curves were established from *E. faecalis* DNA isolated using the Epicentre Masterpure Gram-positive DNA isolation kit (Cambio, Cambridge, UK) and *Candida* DNA isolated using the QIAamp DNA Mini Kit (QIAGEN, Manchester, UK). Both were used in accordance with the manufacturer’s instructions except for one modification, whereby biofilms were scraped off and vortexed for 30 s in initial DNA isolation buffer. After removal from the substratum mixed species biofilms were homogenised and split into two, prior to DNA isolation as described above. The effect of contamination of the target species by the second species was determined by mixing predetermined quantities of biofilm homogenates and comparing with the single species result. The purity
of DNA was checked spectroscopically at 260 and 280 nm in a NANODROP 1000 spectrophotometer (Thermo Scientific, Renfrew, UK) and the ratio of absorbance (260/280) was determined. The amount of DNA in standard samples was measured by a fluorometric analysis using the CYquant assay system (Invitrogen, Paisley, UK). The value was plotted against the number of CFUeq. and these values were utilised to set up standard curves for each microbial assay.

A real-time quantitative polymerase chain reaction (qRT-PCR) assay was used for the detection and quantification of microbial cell numbers or CFU eq. The primers and probes selected for C. albicans (forward primer: CATCCATTTCAGGGCTAGT and reverse primer: CGCTGAACCTAAGCATATCA) and E. faecalis (forward primer: GATGCGCAATTAATCGG and reverse primer: CATAGCCTGTCGCAAAAC; probe: CAATTGGAAAGAGGAGTGGCGGACG (Williams et al. 2006)), were purchased from Invitrogen and Eurogentec (Liege, Belgium), respectively.

qRT-PCR was carried out in a Statagene MRX III thermal cycler (Agilent, Edinburgh, UK). For C. albicans, the cycles with SYBRgreen (ABI, Paisley, UK) were as follows: 10 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C. For E. faecalis, the TaqMAN assay (Invitrogen, Paisley, UK) PCR cycling parameters used were: 10 min at 95°C, 40 cycles of 30 s at 95°C, and 1 min at 60°C.

Primer sets were validated by running four serial 1/10 dilutions of the standard DNA and calculating the efficiency of the reaction (E) where $E = (10^{-1/slope}) - 1$. All reaction efficiencies calculated were between 91% and 104%, and deemed acceptable.

**Tumor necrosis factor alpha (TNF-α) assay**

To assess SEAP validity for determining TLR activation, it was compared to TNF-α levels. Cytokine levels were determined using an enzyme-linked immunosorbent assay (ELISA; Peprotech, London, UK) development kit, which was used according to the manufacturer’s guidelines. The minimum standard used in the assay was 7.9 pg/mL.

**Lactate dehydrogenase (LDH) assay**

To ascertain that all monocytes remained functional despite inflammatory challenge or the root canal irrigants, damage to monocytes was determined by LDH levels in culture media. The assay was performed using the Biovision LDH Kit (Caltagmed Systems, Little Balmer, UK) following the manufacturer’s recommendations. In brief, 10 μL of THP1-XBlue culture fluids were added to 100 μL LDH Reaction mix in a 96-well microtitre plate and incubated for 30 min at room temperature. The reaction was stopped by adding 10 μL of Stop Solution. The absorbance of all controls and samples was determined with a spectrophotometer at 450 nm with
a reference wavelength of 650 nm. As controls, a low LDH sample containing 10 µL of unconditioned medium and a 100% LDH standard containing 10 µL of a THP1-XBlue cell lysate were used. Cytotoxicity was calculated via cytotoxicity (% = \((100 \times (\text{Test Sample} - \text{Low Control})) / (\text{High Control} - \text{Low Control})\).

**Statistical analysis**

The distribution of the data was assessed visually using a dot plot and subjected to log transformation where necessary, i.e., where the data were subject to kurtosis or skewed. Statistical analysis was performed using One-way analysis of variance (ANOVA) with a Bonferroni post hoc test. The level of significance was set to \(P < 0.05\). Statistical analysis and charting of data was performed using GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA). Correlations between SEAP and TNF-α expression were investigated by Spearman’s correlation analysis with IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA).

**RESULTS**

**Stimulation by planktonic microorganisms**

Planktonic bacteria stimulated the immune cells in a dose-response manner, when assessed both by SEAP levels \((r = 0.615; P < 0.001. \text{Figure 5.1a})\) and TNF-α levels \((r = 0.640; P < 0.001. \text{Figure 5.1b})\). Each microorganism significantly stimulated the immune cells as compared to untreated control cells \((P < 0.001)\). SEAP production increased after stimulation, with the positive control *F. nucleatum* and *E. faecalis* having significantly greater gradients in dose-related responses to the bacteria \((P < 0.01)\), followed by the positive control *L. monocytogenes*, the negative control *S. oralis*, and *C. albicans*. *L. monocytogenes* was more stimulatory than *S. oralis* and *C. albicans* \((P < 0.05)\). TNF-α production was also increased after stimulation. *F. nucleatum* and *E. faecalis* had the steepest gradients in a dose-related response to the challenge \((P < 0.01)\), both of which were more stimulatory than *L. monocytogenes*, *S. oralis* and *C. albicans* \((P < 0.01)\). Dose-response curves of SEAP levels were significantly correlated to those of TNF-α levels \((r = 0.780; P < 0.001)\).

In control experiments, MyD88 deficient THP1-XBlue cells did not appear to respond to the tested microorganisms (data not shown). These cells increased SEAP production in response to a NOD1 agonist (L-Ala-γ-D-Glu-D-mDAP), which suggested that the microorganisms stimulated SEAP production only when the MyD88-TLR dependent pathway was available in the THP1-XBlue cells.

THP1-monocytes did not appear to respond to CHX- or NaOCl-treated and untreated planktonic *C. albicans*, and when together with *E. faecalis* (Figure 5.2). The THP-1 cells also did not appear to respond to CHX-treated *E. faecalis*, but
TLR response to root canal pathogens

were stimulated by NaOCl-treated \textit{E. faecalis} (SEAP $P < 0.001$; TNF-$\alpha$ $P = 0.24$) and untreated \textit{E. faecalis} (SEAP $P < 0.001$; TNF-$\alpha$ $P < 0.001$). In addition, SEAP levels were significantly correlated to TNF-$\alpha$ levels ($r = 0.470$; $P = 0.006$).

\textbf{Stimulation by biofilms}

The treated biofilms of \textit{C. albicans}, \textit{E. faecalis} or of their combination did not appear to be capable of TLR activation as assessed by SEAP levels (Figure 5.3a) or TNF-$\alpha$ levels (Figure 5.3b). The untreated \textit{E. faecalis} biofilms elicited a statistically significant increase in SEAP ($P < 0.001$) and TNF-$\alpha$ ($P < 0.001$) production, whereas dual-species biofilms only elicited a statistically significant response when TNF-$\alpha$ levels were assessed ($P = 0.001$). Nevertheless, SEAP levels appeared to increase in parallel and were significantly correlated to TNF-$\alpha$ levels ($r = 0.508$; $P = 0.004$).

The biofilm DNA content was not significantly reduced by either CHX or NaOCl treatment. \textit{C. albicans} biofilms consistently had more DNA than the two other biofilm groups. Moreover, the amount of DNA did not appear to be correlated to SEAP nor TNF-$\alpha$ production or the proportion of dead THP-1 monocytes. Little difference was seen regarding LDH levels in culture fluids of the THP-1 cells stimulated with CHX and NaOCl treated biofilms compared to cells stimulated with the untreated biofilms (Figure 5.3c). Altogether, this implied that reduced SEAP
FIGURE 5.2. TLR stimulation by treated planktonic *C. albicans* and *E. faecalis*. Stimulation of THP1-XBlue cells by planktonic *C. albicans* or *E. faecalis* or both, treated with 2% CHX or 2% NaOCl. Stimulation was assessed by SEAP production (a) and TNF-α production (b). *L. monocytogenes* was supplied with the THP1-XBlue cells and served as a positive control. * Statistically significantly different from control $P < 0.001$

FIGURE 5.3. TLR stimulation by treated biofilms of *C. albicans* and *E. faecalis*. Stimulation of THP1-XBlue cells by biofilms of *C. albicans*, *E. faecalis* or both, untreated and treated with 2% CHX or 2% NaOCl. Stimulation was assessed by SEAP production (a) and TNF-α production (b). Excessive stimulation causing cell death was assessed by proportion of dead monocytes (c). Statistically significantly different from control *$P < 0.001$* or **$P < 0.01$**
or TNF-α levels were not caused by insufficient numbers of microorganisms or a reduction in monocyte viability.

DISCUSSION
Whole root canal samples have been shown to significantly activate TLRs (Martinho et al. 2014). This study showed pronounced TLR activation by E. faecalis, both as a planktonic bacterium and in a biofilm. However, C. albicans separately and also when co-cultured with E. faecalis did not appear to stimulate SEAP or TNF-α secretion by the THP1-XBlue or MyD88 deficient THP1-XBlue monocytes. Planktonic E. faecalis has previously been shown to stimulate TLR2 (Lee et al. 2009), and the degree of stimulation was in a similar range as that for the potent oral pathogen F. nucleatum, which also elicits an immune response via TLR2 (Marcato et al. 2008). C. albicans is associated with disease as well (Schlecht et al. 2015), but barely stimulated TLRs within this study. In comparison to E. faecalis and the positive controls L. monocytogenes and F. nucleatum, the oral commensal S. oralis (Tanner et al. 1998) only slightly stimulated THP-1 cells. The stimulation of SEAP and TNF-a production appears to be due to activation of MyD88 adaptor protein dependent pathways within the THP-1 cell.

The failure of C. albicans to stimulate TLRs did not seem to be due to low numbers of fungi, reduced monocyte viability, or lack of direct fungus to THP-1 cell contact (data not shown). Within a similar in vitro model, planktonic C. albicans was also unable to stimulate TLR2 (Xu et al. 2014). The lack of TLR stimulation does not mean that fungi are not pathogenic. Cell wall glucans are the major fungal PAMPs, primarily recognised by PRRs called C-type lectin receptors (CLRs; Lowman et al. 2014). Moreover, fungi are capable of evading the immune system. The cell wall structure changes continuously during the fungal cell cycle and the morphological conversion between yeasts and hyphae (Lowman et al. 2014). The yeast morphology is associated with a more commensal state of C. albicans and was not expected to elicit a strong immune response. In contrast, hyphae can occur in biofilms and invade host tissues leading to increased mortality (Schlecht et al. 2015).

In contrast to what was expected, the more diverse dual-species infection did not lead to an additional TLR activation, rather the immunostimulatory effects of E. faecalis were reduced in the presence of C. albicans. The interplay between these microbes and with the THP-1 cells can probably occur at several levels and further investigations would be necessary to elucidate the mechanisms. Nonetheless, the interaction at a microbial level will affect the stimulation of the immune system and the subsequent host response. Enterococci adhere to yeast and hyphae of C. albicans (Fox et al. 2014), which leads to a reduced overall biofilm thickness. At the same time, this adherence may shield the E. faecalis PAMPs from recognition by TLR, thereby
decreasing their immunostimulatory potential. Co-infection could also decrease the virulence of both microorganisms. When the gastrointestinal tract of the nematode *Caenorhabditis elegans* is infected with only *C. albicans*, hyphae invade the tissues and kill the worm (Cruz et al. 2013). When it is infected with only *E. faecalis*, the worm dies quickly and merely a portion of the bacteria remain alive. When the worm is infected with both microorganisms, no *Candida* hyphae are formed and all enterococci remain alive. The worm also maintains its intestine integrity and survives longer (Cruz et al. 2013). Our results support this finding that when *E. faecalis* and *C. albicans* are cultivated in combination, the virulence of *E. faecalis* is reduced. However, we were unable to ascertain whether this was also true for *C. albicans*. Thus, the interaction between the microorganisms attenuates their virulence, enabling a commensal relationship with the host. On the contrary, co-infection of *C. albicans* and *S. oralis* within a similar experimental design in an *in vitro* model shows that *C. albicans* neither decreased nor increased the streptococcal TLR2 stimulation (Xu et al. 2014).

The microbial influence can also be directly exerted on the immune system. *C. albicans* can modify the host response and thereby influence TLR activation by *E. faecalis*. For instance, priming cells with *C. albicans* leads to enhanced cytokine production after *in vitro* stimulation with bacteria (Ifrim et al. 2014). Furthermore, TLR activation has been shown to promote tryptophan metabolism, thereby promoting microbial persistence and local immune tolerance and limiting tissue damage (Fallarino & Puccetti 2006; Mahanonda et al. 2007). However, lactobacilli are capable of inducing production of the pro-inflammatory cytokine IL-22, which can activate the immune response against fungi (Zelante et al. 2013). Thus, this complex interaction between microbiota, commensal fungi and the host will construct a delicate balance where TLR activation serves to modulate the host response and allows for interaction with commensal bacteria and fungi to maintain long-term homeostasis. The significance of the interaction between the microbiome and the host in niches which regularly are sterile and whether chronic low grade inflammation has any systemic effects (Van der Waal et al. 2015) still needs to be unravelled.

Despite the potent immunostimulatory effects of *E. faecalis*, these effects were clearly abrogated after treatment with NaOCl or CHX. Lee et al. (2009) have also shown that planktonic *E. faecalis* stimulated immune cells, and that this ability was attenuated when treated with CHX. Although antimicrobials were capable of inactivating the microorganisms in this *in vitro* model, they will not necessarily inactivate all the microorganisms in the biofilm within a root canal system. Both the protective environment of the biofilm and the elaborate extensions of the root canal system will hamper complete microbial removal *in vivo* (Nair et al. 2005). Our results indicated that the antimicrobials diminished the stimulation of the surrogate
root canal biofilm and reduced the effects of the infection on inflammatory cells. However, this would only be effective in vivo if all the microbes in a biofilm were inactivated.

CONCLUSION
The in vitro model used in this study proved helpful in determining the inflammatory response to apical periodontitis pathogens before and after the use of root canal irrigants demonstrated by a reduction of TLR signalling and TNF-α secretion. An additional, but unanticipated result was that the interplay between the two microorganisms and the host resulted in decreased TLR stimulation which may lead to a modification of the innate immune response. In the presence of fungal pathogens, CLR activation and mechanisms downstream are likely to affect the inflammatory response and should be included in further investigations to determine the impact and consequences of PRR activation in the apical tissues.

ACKNOWLEDGEMENTS
The authors have no competing interests relating to this research.

AUTHOR CONTRIBUTIONS
Conceived and designed the study: IFP, WC, MPR, DFL. Performed the study: IFP, GR, DFL. Analysed the data: IFP, DFL. Drafted the manuscript: IFP, DFL. Critically revised the manuscript: WC, MPR, GR. All authors accepted the final version of the manuscript.