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Oral Commensal Bacteria Differentially Modulate Epithelial Cell Death

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Abstract

Objective: Epithelial cell death is an important innate mechanism at mucosal surfaces, which enables the elimination of pathogens and modulates immunoinflammatory responses. Based on the antimicrobial and anti-inflammatory properties of cell death, we hypothesized that oral epithelial cell (OECs) death is differentially modulated by oral bacteria.

Material and Methods: We evaluated the effect of oral commensals *Streptococcus gordonii* (*Sg*), *Streptococcus sanguinis* (*Ss*), and *Veillonella parvula* (*Vp*), and pathogens *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*), and *Fusobacterium nucleatum* (*Fn*) on OEC death. Apoptosis and necrosis were evaluated by flow cytometry using FITC Annexin-V and Propidium Iodide staining. Caspase-3/7 and caspase-1 activities were determined as markers of apoptosis and pyroptosis, respectively. IL-1 β and IL-8 protein levels were determined in supernatants by ELISA.

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Author contributions

Tyresia White contributed to data acquisition, analysis, interpretation, and drafted the manuscript; Yelena Alimova, and Vanessa Tubero Euzebio contributed to data analysis and interpretation; Pinar Emecen, Mohanad Al-Sabbagh, and Jeffrey Ebersole contributed to data interpretation and critically revised the manuscript; Alejandro Villasante developed the statistical analysis; and Octavio A. Gonzalez contributed to conception, design, data analysis and interpretation, and writing of the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Declaration of Competing Interest

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Results: Significant increases in apoptosis and necrosis were induced by *Sg* and *Ss*. *Pg* also induced apoptosis, although at a substantially lower level than the commensals. *Vp*, *Tf*, and *Fn* showed negligible effects on cell viability. These results were consistent with *Sg*, *Ss*, and *Pg* activating caspase-3/7. Only *Ss* significantly increased the levels of activated caspase-1, which correlated to IL-1 β over-expression.

Conclusions: OEC death processes were differentially induced by oral commensal and pathogenic bacteria, with *Sg* and *Ss* being more pro-apoptotic and pro-pyroptotic than pathogenic bacteria. Oral commensal-induced cell death may be a physiological mechanism to manage the extent of bacterial colonization of the outer layers of mucosal epithelial surfaces. Dysbiosis-related reduction or elimination of pro-apoptotic oral bacterial species could contribute to the risk for persistent inflammation and tissue destruction.

Keywords

Oral epithelial cells; oral commensal bacteria; oral pathogenic bacteria; apoptosis; pyroptosis

1. Introduction

The oral epithelium is colonized by a diverse and high number of microorganisms including both commensal and pathogenic bacteria. Fluctuations in the numbers of these bacterial groups are reflected by a continuum ranging from health (symbiosis) to disease (dysbiosis) (Hajishengallis et al., 2011). Among several mechanisms involved in maintaining host-microbe homeostatic interactions at mucosal surfaces, controlled mechanisms of epithelial cell viability and death play a critical role, not only in the physiological and permanent renewal of epithelial surfaces during health and wound healing, but also as an intrinsic immune defense mechanism in response to microbial infections controlling invasive bacteria and viruses (Carvalho-Filho et al., 2013).

Importantly, evidence indicates that strategies used by various bacterial pathogens can manipulate host cell death pathways to enhance their capacity to replicate, survive and disseminate (Lamkanfi & Dixit, 2010). The molecular pathways by which a cell dies (i.e., apoptosis, necrosis, pyroptosis) determine the level of collateral damage and inflammation inflicted on surrounding healthy tissues (Westman et al., 2019). For example, necrosis is considered to be a drastic and uncontrolled form of cell death which necrotic debris are potent inducers of inflammation, delaying the regeneration required after injury and sustaining collateral inflammatory damage (Pasparakis & Vandenabeele, 2015). Pyroptosis is a type of cell death caused by extensive inflammasome activation, marked by caspase-1 activation, and IL-1 β and IL-18 secretion (Bergsbaken et al., 2009). Apoptosis, the prototypical form of programmed cell death, is morphologically described as cell shrinkage and chromatin condensation, followed by fragmentation of the entire cell into smaller, sealed apoptotic bodies. These apoptotic bodies are promptly cleared by neighboring phagocytes, importantly without initiating a pro-inflammatory response or disturbing tissue homeostasis (Westman et al., 2019).

It has been shown that apoptotic cell death is an essential mechanism that regulates the immunoinflammatory response against pathogens, through the generation of anti-

inflammatory signals affecting phagocytes at the site of the infection (e.g., TGF- β), as well as contributing to the characteristics of the T helper and T regulatory (Treg) cell responses (Henson & Bratton, 2013; Nakahashi-Oda et al., 2016). Therefore, the potential for inhibition of apoptosis at epithelial surfaces would increase the likelihood for persistent infection and inflammation, which are central features of periodontal disease. Overall, most of the existing studies involving cell death are primarily focused on periodontopathogenic bacteria, and the role of apoptosis in the pathogenesis of periodontal disease remains uncertain (Gamonal et al., 2001; Jarnbring et al., 2002; Tsuda et al., 2012). Studies suggest that apoptosis may be one mechanism underlying the pathophysiology of periodontal disease progression (Abuhussein et al., 2014; Dabiri et al., 2016). Although the ability to manipulate cell death in oral epithelial cells has been shown for some periodontopathogens such as *P. gingivalis* (Mao et al., 2007; Nakayama et al., 2015; Yao et al., 2010), the impact of other oral bacteria including commensals in oral epithelial cell viability remains limited (Zhao et al., 2019). Moreover, whether oral bacteria selectively and differentially modulate other types of cell death, such as pyroptosis remains unknown.

The historical emphasis on late colonizers of the biofilm overlooks the initial changes and the potential impact of commensal bacteria that occurs earlier in the process of inflammation and infection associated with periodontal disease. A better understanding of the mechanisms by which oral bacteria modulate oral epithelial cells (OECs) death may help to develop new preventive strategies aimed at minimizing the persistent infection and inflammation in the oral mucosa as two central early events in the pathogenesis of periodontitis. Herein, we evaluated the effect of representative oral commensal and periodontopathogenic bacterial species on human OECs death including apoptosis, pyroptosis, and necrosis.

2. Materials & Methods

2.1 Oral epithelial cell cultures

The effect of both oral commensal and periodontopathogenic bacteria in cell death was tested *in vitro*. The immortalized keratinocyte cell line OKF6/hTERT-2 (OECs), established by ectopic expression of the telomerase catalytic subunit in cells from normal oral mucosal epithelium, obtained from Dr. James Rheinwald, Harvard Medical School (Dickson et al., 2000), was used in this study as previously described (Al-Attar et al., 2018; Gonzalez et al., 2013). These cells have normal growth and differentiation characteristics and represent a broadly used model of oral epithelial cell biology allowing for improved reproducibility relative to variation between sources and short lifespan of primary cultures of epithelial cells. OECs were grown in Keratinocyte serum-free media supplemented with 25 μ g/ml Pituitary extract and 0.2 ng/ml epidermal growth factor (Fisher Scientific Company, MA).

2.2 Bacterial cultures

The commensal *Streptococcus sanguinis* ATCC10556, *Streptococcus gordonii* ATCC10558 and *Veillonella parvula* ATCC10790, and pathogenic *Porphyromonas gingivalis* 381, *Tannerella forsythia* ATCC43037, and *Fusobacterium nucleatum* ATCC 25586 bacterial strains were used in the study. All bacterial strains were grown in 5 mL of Brain Heart infusion culture media (BD, Sparks, MD, USA) at appropriate aerobic and anaerobic

atmospheric conditions as previously reported (Al-Attar et al., 2018; Gonzalez et al., 2013; Gonzalez et al., 2010; Huang et al., 2009). *T. forsythia* was grown in ATCC Medium: 1921 NAM medium. Each bacterial strain was grown in 2–3 ml of appropriate broth media to reach logarithmic growth, centrifuged, and resuspended in OEC media (Ker-SFM). Bacterial numbers were determined by count of the cell suspensions using a Petroff Hausser chamber and adjusted to the amounts needed for the corresponding OEC: bacteria ratios to be used in cell death experiments as described in the following section.

2.3 Determination of oral epithelial cell death

OECs (2×10^5 cells/well) were incubated in 6-wells plates at 37°C and 5% CO₂ for 24 hours with either Ker-SFM alone or containing oral bacteria growing at the mid-log phase. Different epithelial cell bacteria ratios (1:10, 1:50, and 1:100) and times (4, 8, 16, and 24 hours) were tested. After bacterial challenge, OECs were harvested by trypsinization and combined with cells rescued from supernatants, washed with PBS and centrifuged at 1100 RPM for 5 minutes. Then, 1×10^5 cells were reconstituted in PBS and labeled with 5 µL of FITC-Annexin V and 5 µL Propidium iodide (BD Pharmingen, San Jose, CA) for 15 minutes at room temperature followed by flow cytometry analysis (FACS). OECs incubated with 16µM staurosporine (Sigma, St. Louis, MO) or only media (Mock) were used as a positive and negative control for apoptosis, respectively. In cells undergoing death process the membrane phospholipid, phosphatidylserine (PS), is translocated from the inner to the outer leaflet, exposing PS to the external cellular environment. With a high affinity for PS, FITC-Annexin V will bind to cells with membrane exposure. Propidium iodide (PI) was added in conjunction with FITC-Annexin V to enable identification of necrotic cells, since PI permeates membranes that are not intact. Annexin V positive but PI negative cells were defined as early apoptotic cells, whereas Annexin V positive and PI positive cells were defined as late apoptotic cells. For each sample 10,000 events were read in a flow cytometer FACSCalibur (Becton Dickinson, San Jose, CA).

2.4 Determination of levels of activated caspases

Activation of caspase-3 is an essential event during apoptosis. Levels of activated caspase-3/7 were determined in OECs by FACS using the Vybrant FAM FLICA Caspase-3/7 Assay Kit (Molecular Probes, Eugene, OR). Levels of activated caspase-1 (pyroptosis marker) were similarly determined using the FAM FLICA Caspase-1 Assay kit (Immunochemistry Technologies, LLC, Bloomington, MN). These systems detect active caspases by use of a fluorescent affinity label which binds covalently with a cysteine that has a specific caspase-amino acid sequence. The fluorescent signal allows for a direct measure of caspase activity (Wlodkowic et al., 2009). After bacterial challenge, cells (attached and from supernatants) were harvested and resuspended in Ker-SFM at a concentration of 1×10^6 cells/ml. Then, 300 µL of cell suspension was transferred to FACS tubes and incubated for 60 minutes with 10 µL FLICA reagent for either activated Caspase 3/7 or activated Caspase 1. Tubes were swirled during incubation for an even distribution of the reagent among the cells. Cells were then washed twice with 1X wash buffer, centrifuged at 1100 RPM for 5 minutes, and pellets resuspended in wash buffer for FACS analysis.

2.5 Determination of IL-1 β and IL-8

Levels of IL-1 β and IL-8 in cell supernatants from unstimulated cells and bacterial-stimulated cells were determined by ELISA (R&D Systems, Minneapolis, MN). The reaction was evaluated at 450 nm in the SpectraMax M2 Spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.6 Statistical Analysis

Statistical analysis included descriptive statistics for the effect of oral bacteria on death of oral epithelial cells. At least 2 independent experiments were done, with each experimental condition performed in duplicate or triplicate. Variations in these continuous variables were compared under normality and equal variance assumptions in un-stimulated cells vs. bacterial-stimulated cells using an Analysis of variance (ANOVA). For significant overall tests, post hoc Fisher's least significant difference (LSD) was performed. Statistical significance was considered at $p < 0.05$. Graphs were made using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla California).

3. Results

The ability of different amounts of oral commensal and pathogenic bacterial species to induce OEC apoptosis and necrosis was first evaluated by FACS using Annexin V and PI staining. Figure 1A shows a representative flow cytometry dot plot for the effects of each bacteria, as well as positive (staurosporine) and negative controls (mock/unstimulated cells). Cells that were double negative (lower left quadrant) represent the percentage of viable cells, Annexin V positive cells (lower right quadrant) represent cells undergoing early apoptosis, double positive cells for Annexin V and PI (upper right quadrant) represents cells in late apoptosis, and cells positive for PI (upper left) denote necrotic cells. The overall effect of different doses of oral bacteria is shown in Figure 1B. The oral Gram-positive commensals *S. sanguinis* and *S. gordonii* induced a significant decrease in the percentage of viable cells along with significant increases in apoptosis and necrosis. In contrast, the oral commensal Gram-negative bacterium *V. parvula* did not affect OEC viability. Among the evaluated pathogens, *P. gingivalis* was the only one that induced any increase in apoptotic cells, although at a significantly lower level compared with the outcomes observed with both oral streptococci. Time course experiments (Figure 2) confirmed the above experiments and showed that only *S. sanguinis* and *S. gordonii* consistently decreased cell viability as early as 4–8h post-challenge, which correlated with significant increases in cells undergoing early and late apoptosis, as well as necrosis (Figure 2A). Likewise, *P. gingivalis* was the only pathogen eliciting any apoptotic events with the major effect seen at 16h post-challenge.

To determine if death of OECs induced by *S. gordonii*, *S. sanguinis*, and *P. gingivalis* was indeed through apoptosis, or perhaps could involve pyroptotic cell death, determination of the levels of activated caspase-3/7 (apoptosis) and caspase-1 (pyroptosis) were evaluated by FACS in OECs exposed to these three bacteria (Figure 3). Consistently, the percentage of OECs with elevated activated caspase-3/7 was increased in OECs exposed to all three bacteria, but not by *F. nucleatum* used as a negative control. Increases in the levels of

activated caspase-1 were seen in OECs exposed to *S. sanguinis* and *P. gingivalis*; however, these increases reached significance only in cells challenged with *S. sanguinis*.

Determination of IL-1 β production by OECs in response to the oral bacteria indicated that only *S. sanguinis*, *S. gordonii* and *P. gingivalis* were able to stimulate the production of this pro-inflammatory cytokine (Figure 4). However, all oral bacteria stimulated the production of IL-8 in a time-dependent manner, which supported the fundamental biological response capacity of the OECs to the bacterial challenge (Figure 4).

4. Discussion

Our findings demonstrated that oral epithelial cell death is differentially induced by oral commensal and pathogenic bacteria, with oral commensals being more pro-apoptotic (*S. sanguinis* and *S. gordonii*) and pro-pyrototic (*S. sanguinis*) than pathogenic bacteria (*P. gingivalis*, *F. nucleatum*, and *Tannerella forsythia*).

Interestingly, *S. sanguinis* induced a strong activation of caspase-1 consistent with IL-1 β release into the supernatants suggesting that OECs exposed to this oral commensal could undergo a pyrototic process. Pyroptosis has been considered an aggressive response of the innate immune system to pathogens, particularly due to its association with three major pro-inflammatory interleukins, IL-1 β , IL-18, and IL-33 (Bortoluci & Medzhitov, 2010). Therefore, some selected oral commensal species could also be contributing to enhance inflammation through dysregulated innate immunity during the pathogenesis of periodontal disease linked to the ability to stimulate the production of pro-inflammatory cytokines, as has been shown in other streptococcal pathogenic species (LaRock & Nizet, 2015).

Caspase-3/7 was also activated by *S. sanguinis*. This is consistent with accumulating evidence suggesting that there seems to be cross-talk between cell death pathways where caspase-1 could proteolytically cleave (i.e. activate) other caspases involved in apoptosis (Miao et al., 2011).

Although *S. gordonii* did not activate caspase-1, the levels of IL-1 β were elevated in OECs exposed to *S. gordonii* as previously shown by others in both OECs and monocytic cells (Ciabattini et al., 2006; Dickinson et al., 2011; Kesavalu et al., 2002). The mechanisms by which *S. gordonii* induces IL-1 β production may be caspase-1 independent.

One mechanism by which apoptosis can function without inducing inflammation involves the production of TGF- β by apoptotic cells, which regulates the production of inflammatory mediators (Huynh et al., 2002). Most recently it has been shown that prostaglandin E2 (PGE2) is elevated in dying cells and PGE2 can exhibit significant anti-inflammatory properties (Hangai et al., 2016). Thus, increased levels of TGF- β and PGE2 produced by oral epithelial cells undergoing apoptosis after exposure to *S. gordonii* or *S. sanguinis* is a plausible mechanism by which pathologic inflammation could be modified by oral commensals. In addition, evidence indicates that both *S. sanguinis* and *S. gordonii* have the ability to produce hydrogen peroxide (H₂O₂), which has been shown to correlate with increased cell DNA fragmentation and caspase activity, both events that could be activating

the intrinsic pathway of apoptosis (Li et al., 2016; Okahashi et al., 2014). The mechanisms by which these oral streptococci induce epithelial cell death require further study.

A recent study showed that inhibition of IL-8 up-regulated the expression of pro-apoptotic factors while downregulating anti-apoptotic factors simultaneously in endothelial cells (Choi et al., 2016). Consistently, IL-8 silencing decreased Bcl-2 expression in cancer cells with a consequent increase in apoptosis (Stronach et al., 2015). Similarly, herein *S. sanguinis* and *S. gordonii* significantly decreased the release of IL-8 after eight hours of infection, which was consistent with an increase in late or early apoptosis, respectively. The biological relevance and mechanisms involved in cell death associated with variations of chemokine signal expression would require future investigation.

Both apoptotic and pyroptotic cell death types contribute to removal of cells that have been infected by invasive bacteria such as *P. gingivalis*. Thus, a significant decrease in oral commensal species with the ability to induce cell death during dysbiosis, could improve the environment for pathogens like *P. gingivalis* to persist and orchestrate epithelial barrier damage and chronic inflammatory responses (Darveau, 2010). Oral commensal bacterial-induced OEC death could be an additional protective mechanism resulting from normal bacterial colonizers, such as *S. gordonii*, through which epithelial responses are modulated to limit inflammation and disease triggered by pathogens like *P. gingivalis* (Ohshima et al., 2019).

P. gingivalis enhanced apoptosis in OECs, although at a significant lower level compared with the effects found with commensal streptococci. These results are consistent with research by Stathopoulou et al. (Stathopoulou et al., 2009), where live *P. gingivalis* caused apoptosis in human epithelial cells using similar experimental conditions. Importantly, gingipains, the cysteine proteases produced by *P. gingivalis*, were directly involved in these responses. In addition, increased levels of IL-1 β and caspase-1 were seen in OECs exposed to *P. gingivalis*, which indicates that this periodontopathogen could have the ability to induce oral epithelial cell death through pyroptosis, as has been shown in other cell types, *i.e.* monocytic cells (Park et al., 2014). This is also consistent with the ability of *P. gingivalis* to activate the inflammasome, a complex formed by a NOD-like receptor, an adaptor protein (ASC), and caspase-1 (Hung et al., 2013). These findings contrast with a previous report showing that IL-1 β was not detected in OEC supernatants after *P. gingivalis* challenge (Dickinson et al., 2011). This variation could be related to differences in the experimental model and approaches used, since Dickinson's group used organotypic cultures of primary human OECs.

The observation of apparent necrotic cell death in response to some of the oral bacteria (*S. sanguinis*, *S. gordonii*, and *P. gingivalis*) needs to be further defined since it could be secondary to apoptosis in the absence of phagocytosis (Krysko et al., 2008). Historically, necrosis is defined as a mode of cell death that resulted from abrupt changes in noxious stimuli, such as temperature and pressure. The lysed cellular contents would lead to inflammation through receptor activated release of cytokines, chemokines, and adhesion molecules (Yang et al., 2015). Normally, apoptotic cells within tissues are phagocytosed by macrophages without substantial inflammatory responses; however, since our experiments

were developed using an *in vitro* system, the identified necrotic events could be secondary to the lack of physiologic phagocytic processes, and not reflecting a true bacterial-driven necrosis.

Of interest was that the oral bacterial species *V. parvula*, *F. nucleatum* and *T. forsythia* did not have a significant effect on oral epithelial cell viability. Previous studies have shown pro-cell death and pro-inflammatory properties of some of these bacteria in other cell types (e.g., neutrophils and mononuclear cells) (Bhattacharya et al., 2014; Jewett et al., 2000; Karlsson et al., 2002; Settem et al., 2012). Thus, cell death responses appear to be differentially induced and regulated by oral bacteria specifically related to the cell type. Moreover, enhancement of inflammatory responses may depend upon the need for direct contact with innate immune cells following epithelial barrier disruption. Accordingly, the preservation of the epithelial barrier integrity would be presumed to be critical in avoiding pathologic inflammatory responses induced by some oral bacterial species. Likewise, *in situ* synergistic cooperation among bacterial complexes may alter the presence or magnitude of a cell death response with a mono-infection challenge with these specific oral bacterial species (Inagaki et al., 2006).

Since oral commensal bacteria are more abundant in health and even disease microbial ecologies, oral epithelial cell death (either pyroptosis or apoptosis) could reflect a physiological mechanism, by which oral epithelial surfaces can more effectively manage the bacterial burden. The anti-inflammatory properties associated with apoptotic processes could also be critical for maintaining a balanced and homeostatic environment to avoid pathologic inflammation. Reduction or elimination of pro-apoptotic oral bacterial species during dysbiosis could lead to persistent inflammation and tissue destruction in the pathogenesis of periodontal disease. A better understanding of the mechanisms by which oral bacteria differentially modulate OEC death and survival will help to develop new disease management strategies aimed at controlling the persistent infection and inflammation in the oral mucosa, which are two central events involved in the pathogenesis of periodontal diseases. Future studies evaluating additional cell markers to distinguish specific death processes and *in vitro* and *in vivo* experimental strategies to validate these findings appear warranted as the field attempts to distinguish the molecular role of commensal bacteria as part of the innate immune capabilities.

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Highlights

- Epithelial cell death is a regulatory process of inflammation and tissue homeostasis
- Oral commensal bacteria differentially regulate epithelial cell death
- Some oral commensal bacterial species likely contribute to oral inflammation
- Oral epithelial cell death appears to be poorly induced by periodontopathogens

Figure 1A

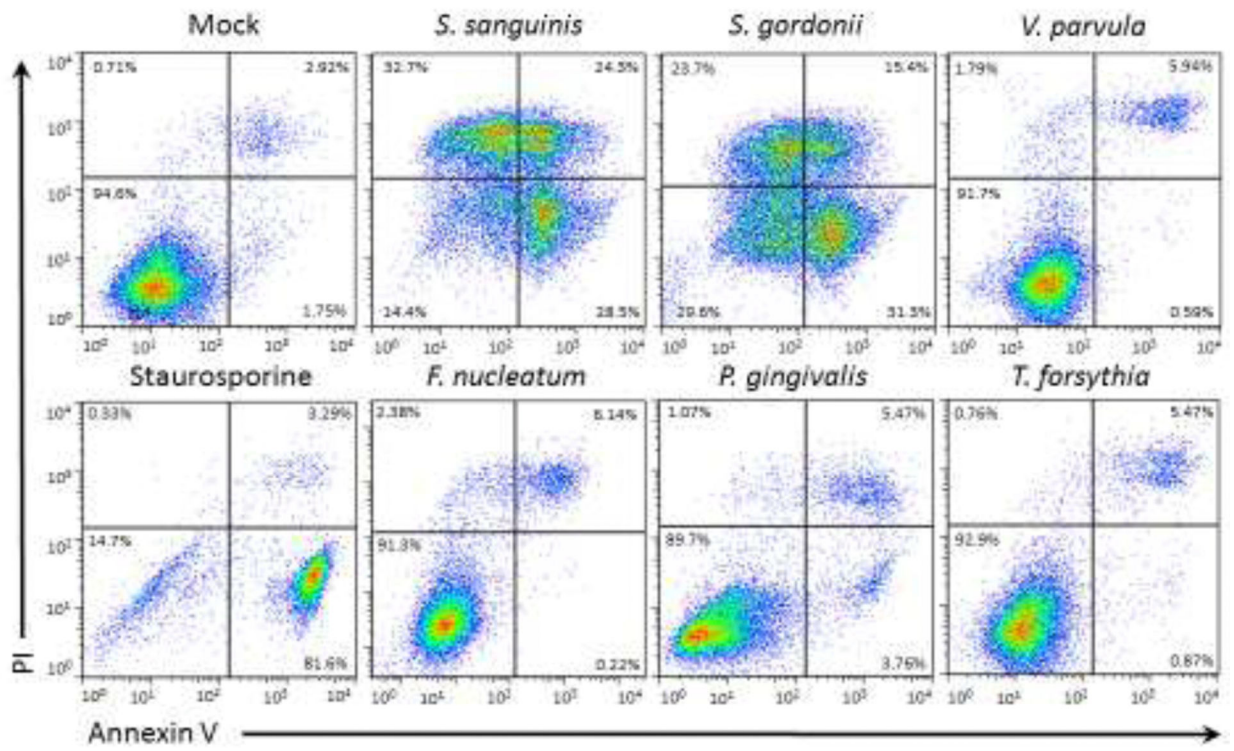


Figure 1B

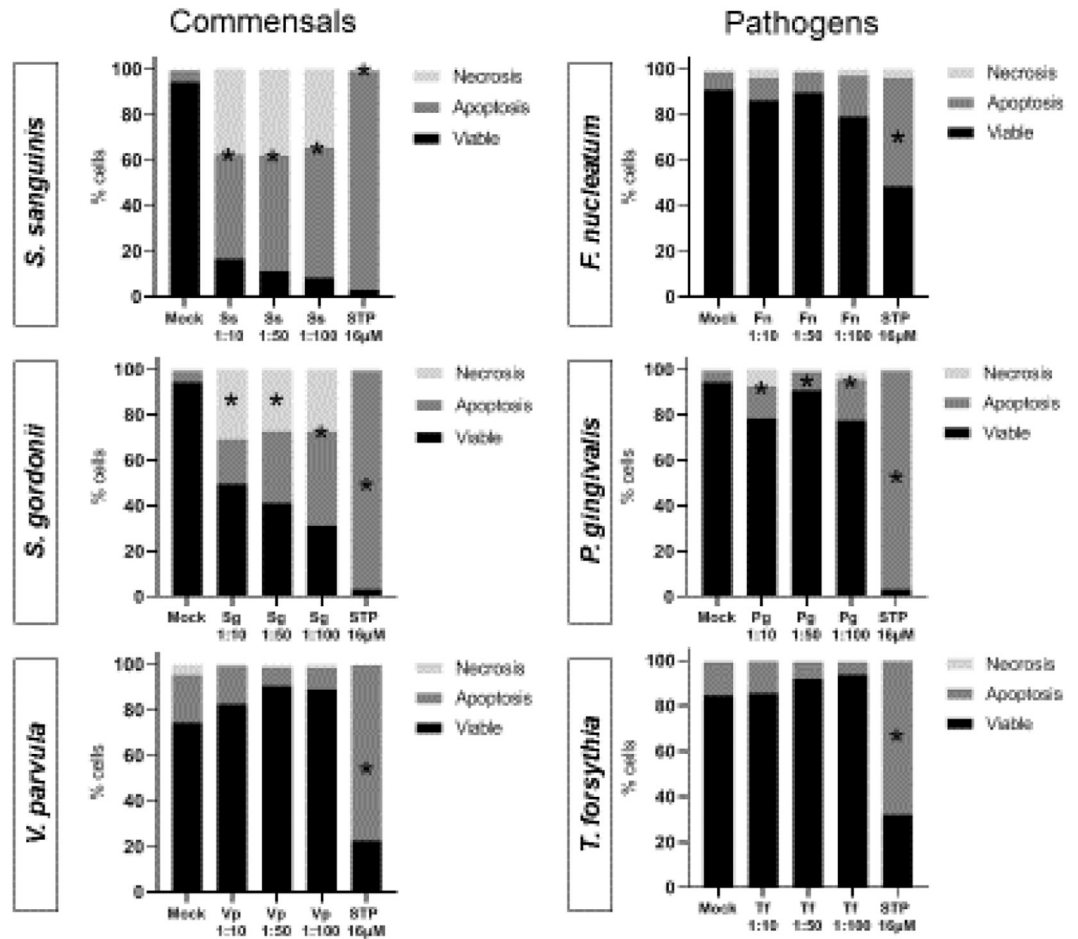


Figure 1. Effect of oral commensal and pathogenic bacteria on epithelial cell death (Dose-response). (A) Representative dot plots and (B) mean of percentages for viable cells or undergoing cell death (apoptosis or necrosis) after 24h bacterial challenge of two independent experiments analyzing at least 10,000 events by FACS for each condition is shown. Viable cells (Annexin V⁻/PI⁻), early apoptosis (Annexin V⁺), late apoptosis (Annexin V⁺/PI⁺), and Necrosis (PI⁺). STP: 16 μ M Staurosporine was used as a positive control. *Treated cells vs. Unstimulated (Mock) cells $p < 0.05$ (ANOVA and post hoc Fisher LSD).

Figure 2A

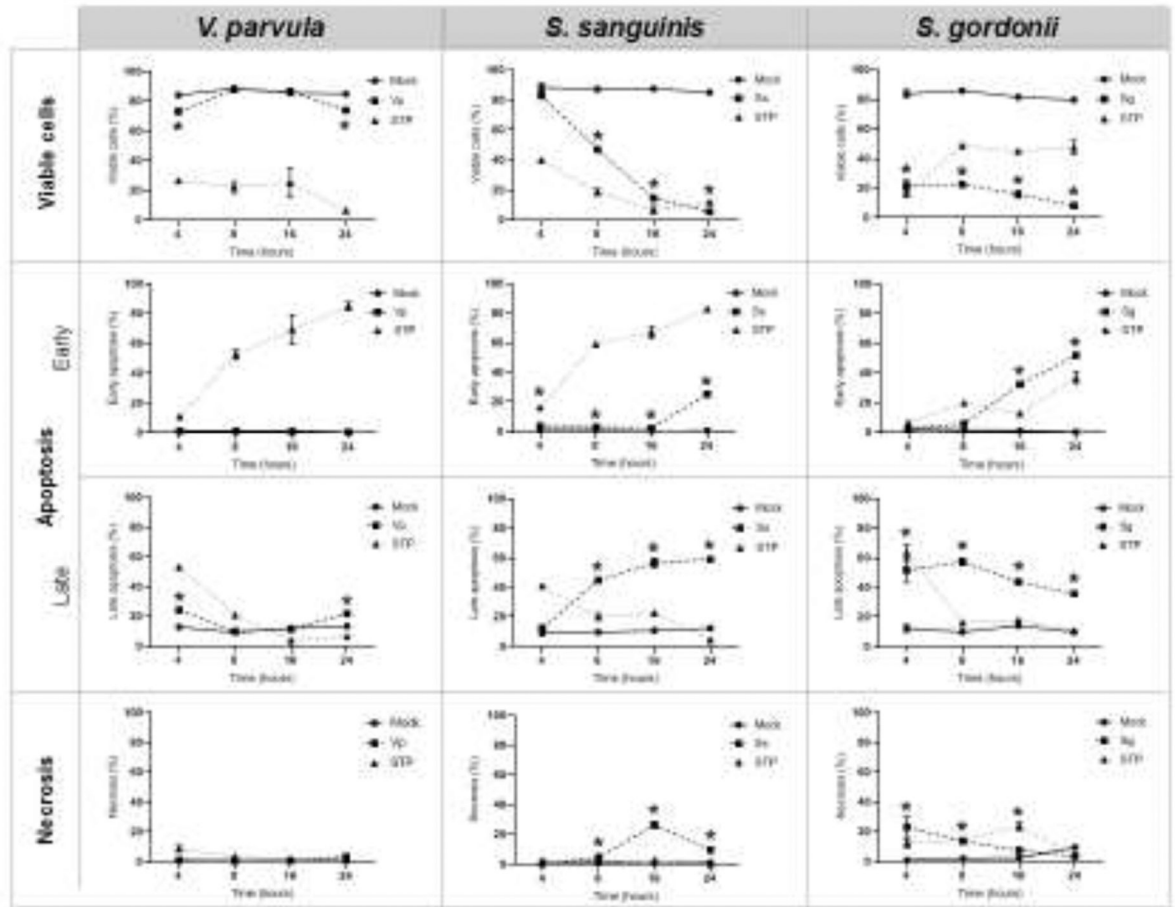


Figure 2B

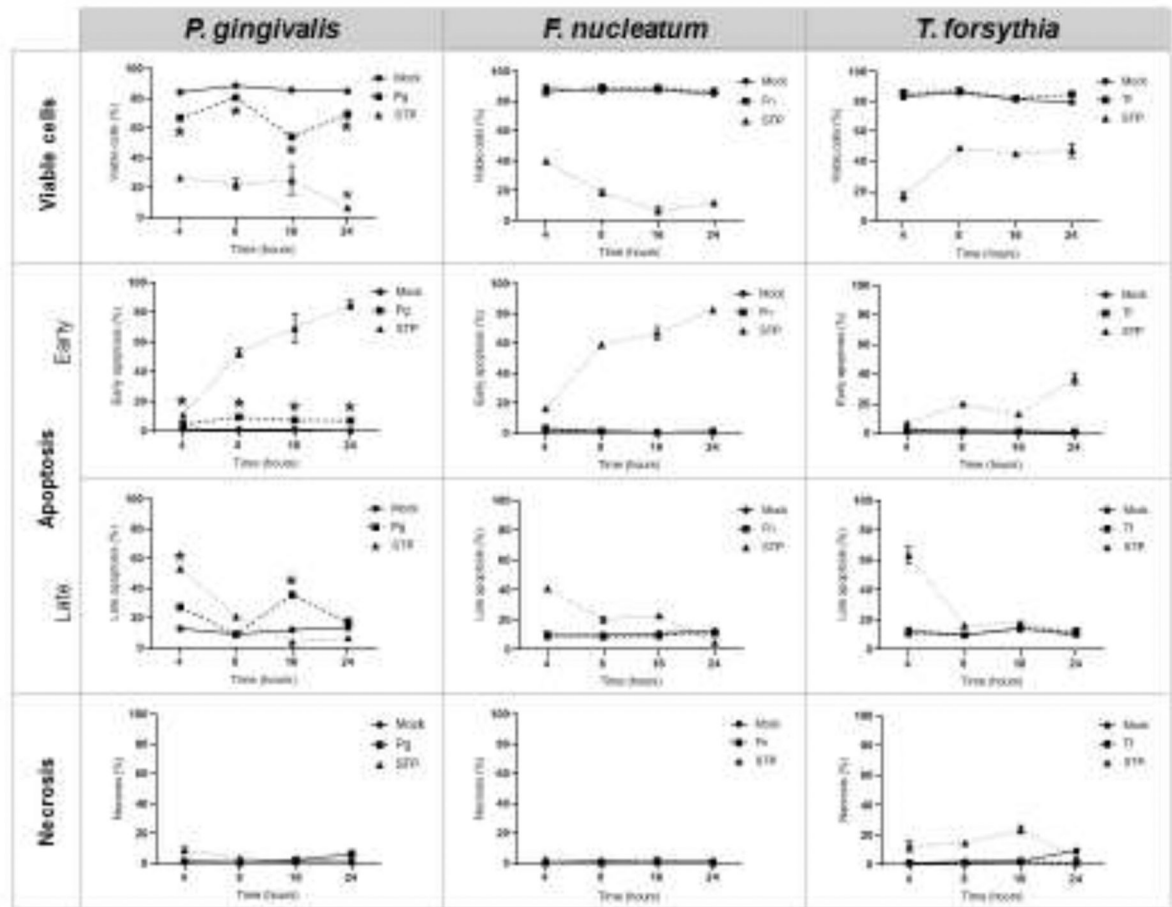


Figure 2.

Effect of oral (A) commensal, and (B) pathogenic bacteria on epithelial cell death (Time-response). MOI [1:50] was used for all bacteria. For each condition, at least 10,000 events were analyzed by FACS to identify viable cells (Annexin V⁻/PI⁻), or undergoing early apoptosis (Annexin V⁺), late apoptosis (Annexin V⁺/PI⁺), and Necrosis (PI⁺). STP: 16 μ M Staurosporine was used as a positive control. The mean \pm standard deviation from two independent experiments with each condition in duplicate is shown. *Treated cells vs. Unstimulated (Mock) cells $p < 0.05$ (ANOVA and post hoc Fisher LSD).

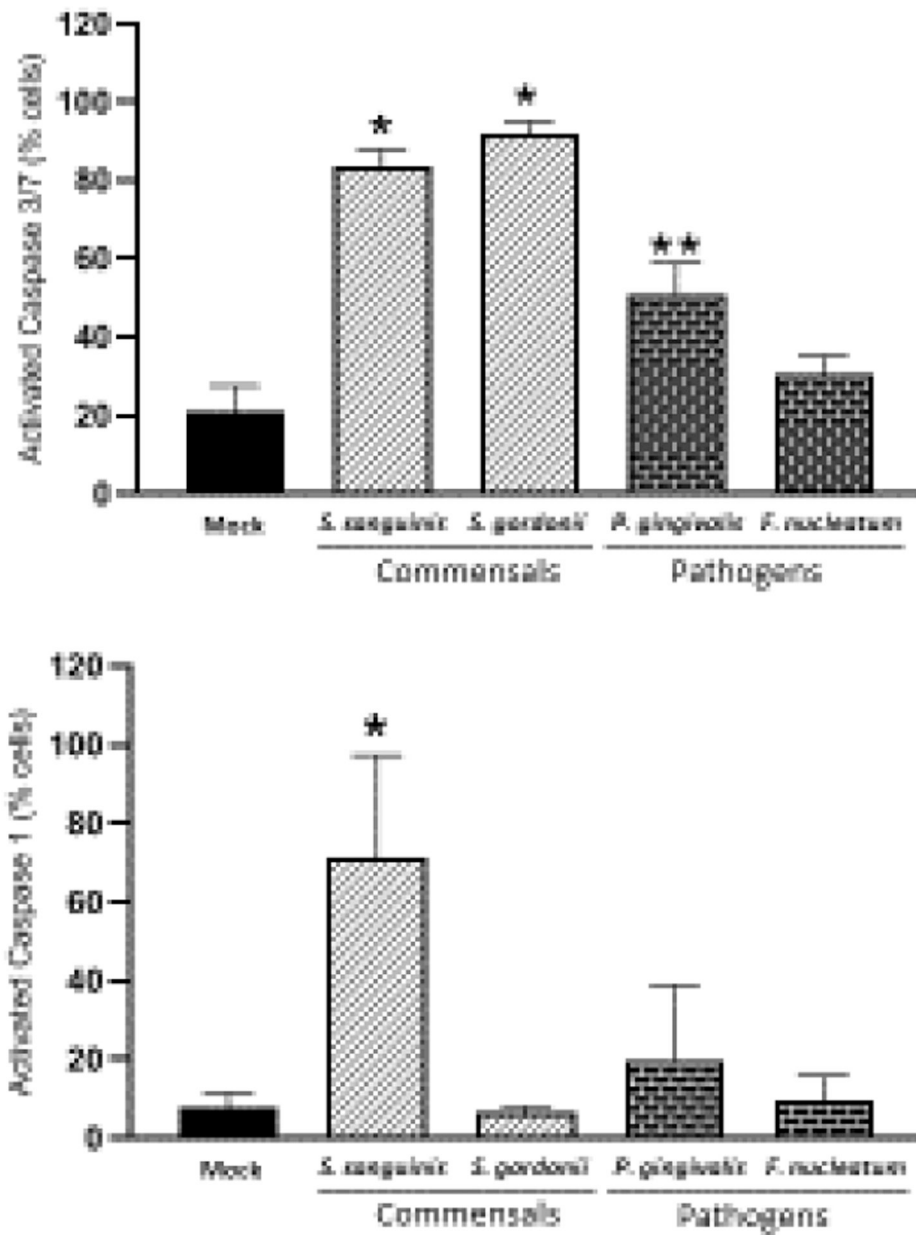


Figure 3. Effect of oral commensal and pathogenic bacteria on Caspase 3/7 (apoptosis) and Caspase-1 (Pyroptosis) activation in epithelial cells after 24h. Mean and standard deviation bars of percentage of cells expressing activated Caspase-3/7 or activated Caspase-1 for each condition are shown. For each condition 10,000 events were analyzed by FACS. The mean \pm standard deviation from two independent experiments with each condition in duplicate is shown. MOI=1:50 *Treated cells vs. Unstimulated (Mock) cells * $p < 0.01$; ** $p < 0.05$ (ANOVA and post hoc Fisher LSD).

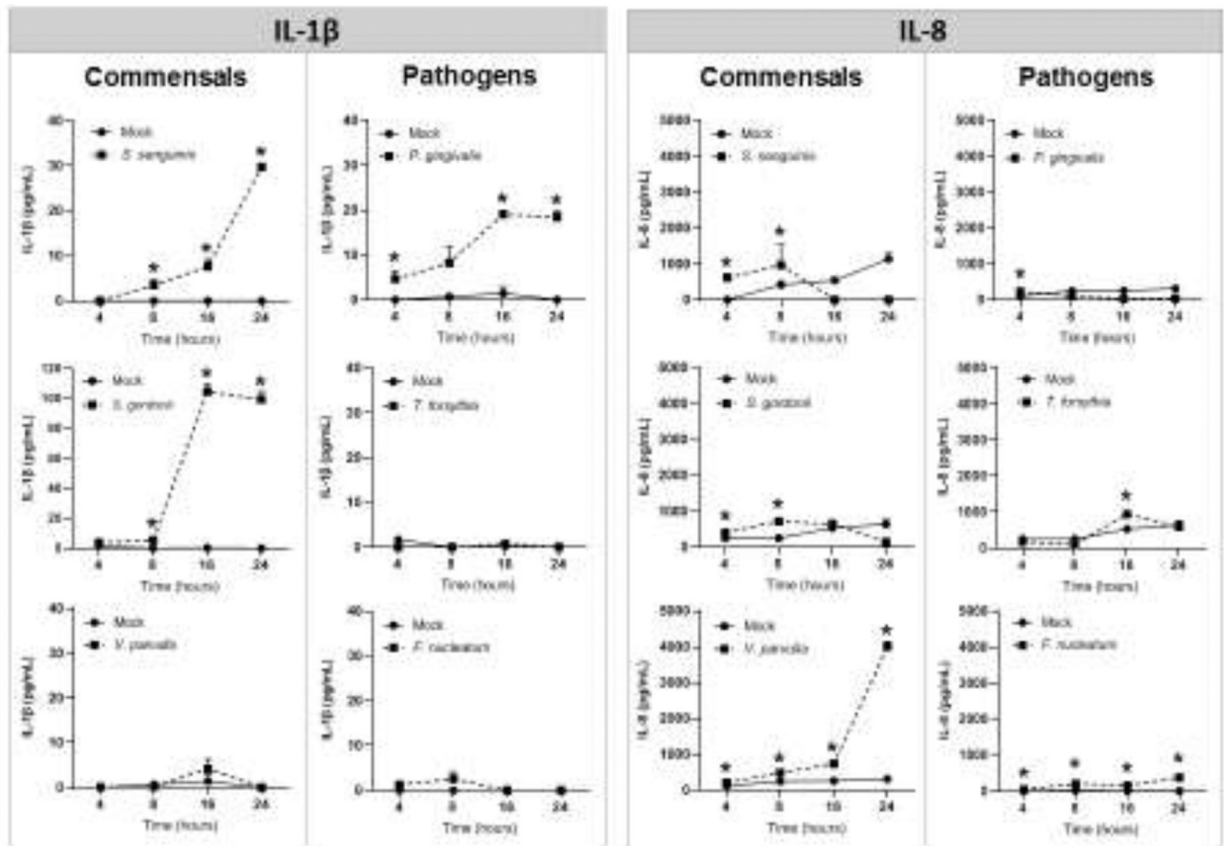


Figure 4.

Effect of oral commensal and pathogenic bacteria [MOI=1:50] on IL-1 β and IL-8 production in oral epithelial cells. Mean \pm standard deviation bars of cytokine levels (pg/ml) detected by ELISA in cell culture supernatants from two independent experiments for each condition in duplicate is shown. *Treated cells vs. Unstimulated (Mock) cells $p < 0.05$ (ANOVA and post hoc Fisher LSD).