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S. gordonii-PRODUCED HYDROGEN PEROXIDE MODULATES miR-663A AND CCL20 EXPRESSION IN ORAL EPITHELIAL CELLS

Thesis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at the College of Medicine at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2021

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ABSTRACT

S. gordonii-PRODUCED HYDROGEN PEROXIDE MODULATES miR-663a AND CCL20 EXPRESSION IN ORAL EPITHELIAL CELLS

The mechanisms through which a persistent recognition of commensal bacteria by oral epithelial cells (OECs) mitigates an uncontrolled inflammatory response of the oral mucosa remain unknown. CCL20 secretion by OECs in response to pathogenic bacteria is regulated by *S. gordonii (Sg)*-induced miR-663a; nevertheless, the mechanisms involved in these *Sg*-modulated responses remain to be elucidated. Since *Sg* is a hydrogen peroxide (H_2O_2) producer, and H_2O_2 has been shown to stimulate miRNA expression, we hypothesized that H_2O_2 could be involved in *Sg*-induced miR663a and CCL20 responses. Expression of miR663a was stimulated by *Sg* and H_2O_2 in a dose-dependent manner. Response was significantly attenuated by catalase. Strong miR663a up-regulation was associated with H_2O_2-producing oral streptococci. Catalase treatment rescued *An*-induced CCL20 expression attenuated by *Sg* and *Ss.* Here we showed that H_2O_2 produced by *Sg* and other oral streptococcal species is involved in miR663a up-regulation and regulation of CCL20 secretion by OECs. H_2O_2-producing oral streptococci could represent a group of commensal bacteria contributing to attenuating oral inflammation and dysbiosis.

Keywords: oral epithelial cells, *S. gordonii*, microRNA, chemokine, hydrogen peroxide, dysbiosis

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S. gordonii-PRODUCED HYDROGEN PEROXIDE MODULATES miR-663A AND CCL20 EXPRESSION IN ORAL EPITHELIAL CELLS

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I would like to dedicate this to my mother, Judy Maynard, a person who was my anchor during my master's degree, who is now watching over me in Heaven

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Chapter 1: Oral Microbial Ecology

Diverse microorganisms inhabit the oral cavity and are unique to this niche as they have evolved an exquisite specificity for oral colonization [1-3]. Within the oral cavity, there are distinct micro-environments such as the hard non-shedding surfaces of the teeth and the epithelial surfaces of the mucosal membranes [4]. In addition to microbial composition, the spatial and structural organization of natural microbial communities is being increasingly recognized as essential for physical and metabolic interspecies interactions that can be antagonistic or cooperative [5, 6]. Gingivitis is an almost inevitable consequence of prolonged accumulation of biofilms (plaque) on tooth surfaces; it is a controlled immune-inflammatory state that does not permanently compromise the integrity of the tissues supporting the teeth [4].

Under some conditions (*e.g.,* immunodeficiencies, smoking, pathogens, diet), the host-bacterial community interaction becomes dysbiotic and irreversible tissue destruction of bone and gums (gingivae) occur (i.e., periodontal disease) [7-9]. Thus, periodontal disease is a chronic inflammatory disease triggered by bacteria with local (*i.e.*, tooth loss) and systemic effects (*e.g.*, diabetes and cardiovascular disease) if not treated. [10-12]. Periodontitis is widely regarded as the second most common type of dental disease worldwide, following dental caries. About 40-50% of the population in the United States has periodontal disease in moderate and severe forms [13-15]. Therefore, a better understanding of the host-bacteria interactions involved in oral health that prevent the transition

from oral symbiosis to dysbiosis is needed for the development of future strategies to prevent/treat periodontal disease.

Most of the studies related to the pathogenesis of periodontal disease have been focused for decades in the identification of oral bacterial species associated with disease [16]. It is currently accepted that specific oral anaerobic bacterial species (e.g., *P. gingivalis, T. forsythia, T. denticola, F. nucleatum*) are causative agents of periodontitis, based on the virulence properties and strong association with diseased sites [17]. Thus, the microbiota associated with a healthy state is considered more “generalist”, whereas the disease-associated microbiota is influenced by ‘specialist’ microorganisms that possess metabolic functions and an elevated virulence potential that are largely absent in health [18].

The distinction between pathogens and commensals is becoming increasingly blurred, especially in diseases that ensure from the action of bacteria that are also present in health and that involve complex host-microbe interactions, as human periodontal disease [19]. Using whole genomic DNA probes and checkerboard DNA-DNA hybridization, Socransky and colleagues characterized periodontal microbial communities based on a color-coded system that reflected cluster analysis, community ordination and associated disease severity [20]. The convenience and appeal of the concept of “red” and other color-coded complexes led to widespread adoption up to present day [19]. Socransky and Haffajee, et al., proposed that oral diseases could be better understood by focusing on the consortia of organisms rather than on individual pathogens. They suggested that the most pathogenic complex comprised *Porphyromonas gingivalis (P. gingivalis),*

T. forsythia, and *Treponema denticola* in the red complex and depended on earlier colonization of the pocket by a complex of somewhat less pathogenic organisms [20].

As molecular-based approaches to microbe detection became increasingly facile, and as studies, using culture-independent methodology for analysis of the periodontal microbiota became abundant, two newer concepts emerged. First, red complex organisms such as *P. gingivalis* can be found in the absence of disease, further argument against the organisms as classical exogenous pathogens [21-23]. Second, the periodontal microbiota is more heterogeneous and diverse than previously thought [24-26], with over 700 bacterial species recognized as possible components, of which around 200 can be present in any one individual and about 50 are present at any one site [27]. Many of these newly recognized organisms show as good or better a correlation with disease as the classical red complex [2, 28]. Recent studies have demonstrated that specific "key" stone pathogens (e.g., *P. gingivalis*) normally found in the oral microbiota can trigger significant changes in the abundance of bacterial species normally found in oral health, which is defined as oral dysbiosis. Recent data from metagenomic, metatranscriptomic, and mechanistic studies are consistent with a new model of periodontal disease pathogenesis, which suggests that a more diverse periodontitis-associated microbiota is involved in the disease than previously thought [1, 2, 18, 24, 29-31]. Periodontal disease results, not from individual pathogens but rather from polymicrobial synergy and dysbiosis, which perturb the ecologically balanced biofilm association with periodontal tissue homeostasis [19, 32, 33].

According to Nishihara and Koseki, the conversion from periodontal health to disease is accompanied by a shift in indigenous microflora of plaque biofilm from gram-positive facultative bacteria to gram-negative microorganisms [34]. It is now recognized that a pathogenic/dysbiotic biofilm is a prerequisite for periodontitis to develop but is insufficient to cause the disease. The disease results from complex interactions between the biofilm and the inflammatory immune response, and it is the latter that is estimated to account for almost 80% of the risk of periodontal tissue damage [35, 36]. A common change in the oral microbiota in diseased sites, gingivitis, and periodontitis, is a decrease in the proportions of health-associated bacteria such as Streptococcus and an increase in anaerobic proteolytic bacteria, *P. gingivalis, T. forsythia, and T. denticola* [1, 19].

Of note, despite there being significantly higher numbers of commensal bacteria in the microbial ecology, and even biofilms at the sites of periodontal lesions, significantly fewer studies have emphasized the important role of commensal bacterial species in maintaining oral health or contributing to a disease process. The mechanisms through which the oral epithelial inflammatory responses to commensal bacteria are regulated to maintain homeostasis and mitigate breakdown of the epithelial barrier and further tissue inflammation and damage remain unclear.

Chapter 2: Dysbiosis

It has been recently demonstrated that a variation in the abundance of bacterial species normally observed in oral health (i.e., dysbiosis), is a critical step for the development of oral pathologic inflammation in periodontal disease [19]. Factors that can enhance oral dysbiosis could be environmental factors (*e.g.*, smoking), host factors (*e.g.,* diabetes), and specific pathogens (*e.g.*, *P. gingivalis*). The relation between dysbiosis and oral inflammation suggests that there could be oral bacterial species that play a critical regulatory role of inflammation to maintain homeostasis. This could be directly modulating the abundance of other pathogenic bacterial species through antibacterial mechanisms (e.g., bacteriocins) or indirectly modulating host mucosal inflammatory responses. Nevertheless, identification of such anti-inflammatory oral bacterial species and the potential cellular and molecular mechanisms involved in regulating inflammation remain to be determined.

Chapter 3: Hydrogen Peroxide-Producing Streptococcus

The genus *Streptococcus* is the most abundant in the salivary microbiome, regardless of the geographic origin of the saliva sample, and accounts for 22.7% of all sequences detected during microbiome sequencing [37]. For example, *Streptococcus salivarius (S. salivarius)*, *Streptococcus sanguinis (S. sanguinis)*, *Streptococcus parasanguinis (S. parasanguinis)*, *Streptococcus gordonii (S. gordonii)*, and *Streptococcus australis (S. australis)* are found on multiple soft surfaces within the cavity. In contrast, *Streptococcus intermedius (S. intermedius)* and *Streptococcus constellatus (S. constellatus)* are associated with dental plaque on tooth surfaces and their presence is implicated in the development of periodontal diseases [20].

The mitis group of streptococci**,** a group of early oral colonizers usually precedes the multiplication of the predominately gram-negative, biofilm producing colonizers [38-40]. Primary colonizers of the mitis group streptococci have adhesin molecules that recognize and bind to complementary salivary receptors that provide receptors for secondary colonizers, later forming biofilms [40, 41]. Other bacterial species of mitis group include specialized pathogens such as *Streptococcus pneumoniae (S. pneumoniae)*, and less pathogenic streptococci like *Streptococcus mitis (S. mitis)*, *Streptococcus oralis (S. oralis)*, *S. sanguinis*, or *S. gordonii* [42].

A feature of streptococcal species of the mitis group is that produce hydrogen peroxide (H_2O_2), which is considered to play an important role on bacterial competition in microbial communities [43, 44]. H_2O_2 is the simple

peroxide, and stronger oxidizer, which is linked to its cytotoxic and tissue-damaging effects [45, 46]. The cytotoxicity and tissue damaging effects of H_2O_2 may be associated with pathogenic properties of the mitis group of streptococci. Therefore, it is likely that streptococcal H_2O_2 enables bacteria to escape from macrophage phagocytosis, and damages epithelial barriers, thereby contributing to bacterial dissemination [47]. *S. gordonii* and *S. sanguinis* are members of the mitis oral streptococci that produce H_2O_2 as a competition mechanism to keep microbiota homeostasis and fight against foreign pathogens from growing [48]. *Streptococcus mutans (S. mutans)* and *S. salivarius* are also members of the oral commensal bacteria, and they do not produce H_2O_2 [47, 49]. H_2O_2 is better known for its cytotoxic effects, in recent years it has become established as an important regulator of eukaryotic signal transduction [50]. H_2O_2 is generated in response to various stimuli, including cytokines and growth factors, and is involved in regulating biological processes as diverse as immune cell activation and vascular remodeling in mammals [51, 52].

H_2O_2-producing streptococcus contain the pyruvate oxygenase gene (*spxB*). *SpxB*-dependent H_2O_2 generation is conserved among most of the oral streptococci and is typically expressed as a single gene controlled by its own promoter [53]. When comparing the aerobic growth of H_2O_2 -producing streptococci in the presence and absence of externally added catalase, it is evident that H_2O_2 production yields self-toxicity [54]. The absence of catalase or other commonly employed H_2O_2 detoxifying enzymes in oral streptococci also suggests

that other important, perhaps uncharacterized; mechanisms are involved in protection from H_2O_2 toxicity [53].

Okahashi et al., demonstrated that H_2O_2 acts as a cytotoxin that contributes to the virulence of the mitis group of streptococci [47]. Likewise, some bacterial species such as *Actinomyces naeslundii (A. naeslundii)* can produce catalase, a H_2O_2-decomposing enzyme, which has been shown to reduce oral epithelial cell death [47]. There is a considerable variation among cell types in the concentration of exogenous hydrogen peroxide required to initiate a particular biological response. For example, the concentration of hydrogen peroxide required to cause apoptotic cell death of mammalian cells can vary as much as 20-fold, depending on the cell type [55-57]. Recent studies in mammalian cells have revealed concentration-specific responses to H_2O_2. For example, different patterns of p53-regulated gene expression are initiated in response to different H_2O_2 levels (0.2 mM, 0.4 mM, and 1 mM) , antioxidants are induced in response to low levels of H_2O_2, where they lower the reactive oxygen species (ROS) levels and hence, protect the cell from DNA damage, while higher levels also stimulate the expression of pro-oxidants involved in apoptosis [57].

H_2O_2 is now thought to influence signaling pathways that modulate some proinflammatory responses [58-60]. A study conducted by Okahashi et al., found that infection with viable *S. oralis*, a H_2O_2 producer, or exposure to 1 mM H_2O_2 induced IL-6 production in epithelial cells. IL-6 is a pleiotropic pro-inflammatory cytokine that acts on various cells [61, 62] that promotes the differentiation of B- and T-cells, thus amplifying immune and inflammatory responses [47]. Several

studies have demonstrated that subtoxic levels of H_2O_2 and other ROS stimulate the production of IL-6 in epithelial cells [58, 63, 64]. Hydrogen peroxide is thought to differentially regulate the expression of IL-6 and β-defensin 2 in epithelial cells [47]. Oral mitis group of streptococci can stimulate cytokine and defensin productions in epithelial cells [65-67]. Ji, S., et al. reported that viable *S. sanguinis* enhanced IL-1alpha production in human gingival epithelial cells. They showed that *S. sanguinis* did not induce β-defensins and cathelicidin expression [65]. Hasegawa, Y., et al. described that viable *S. gordonii* inhibits IL-6 and IL-8 secretion from gingival epithelial cells [66]. Infection with these oral streptococci seem to evoke multiple effects on epithelial cells including the cytotoxicity of H_2O_2 [47].

Beyond the conventional view of ROS as drivers of inflammation, it has been recently demonstrated that H_2O_2 released by streptococcal species inhibits the inflammasomes-dependent innate immunity as a mechanism that bacteria could use to restrain the immune system to co-exist with the host [68]. Inflammasomes are vital players in the eradication of bacterial pathogens [69-71]. Inflammasomes are intracellular multiprotein complexes that control the activation of Caspase-1, mediating pyroptotic cell death and the maturation of IL-1 family cytokines that are essential for optimal defense against pathogens [69, 71]. Generally, inflammasome activation involves two checkpoints: a priming step to induce the synthesis of pro-IL-1β and certain inflammasome components and a second step triggering the assembly and activation of inflammasome complexes [72].

Correspondingly, recent studies also reveal that an increasing number of pathogens are equipped with mechanisms to circumvent inflammasome activation [73-75]. Such pathogens can overcome inflammasome-dependent host defenses either by evading receptor recognition or though active mechanisms of inflammasome suppression [74]. The active mechanisms on inflammasome suppression reported thus seem to require an intimate host-pathogen contact to permit the delivery on inhibitory effectors into the host cells [73-75]. Arguably, such directed delivery of virulence factors is probably essential to ensure that immune suppression is not global but only limited to the infected and not the healthy bystander cells [68].

Chapter 4: Macrophage Inflammatory Protein-3 Alpha (MIP3α)

Oral dysbiosis normally leads to an inflammatory response in the gingival tissue through the activation of prostaglandins, cytokines, and chemokines [76, 77]. The levels of the pro-and anti-inflammatory cytokines in specific sites and at various time points, affect the control of initiation, progression, and inhibition of inflammation [78]. Chemokines are also involved in both physiology and pathology of bone metabolism as chemokines are essential signals for trafficking osteoblast and osteoclast precursors as well as act as potential modulators of bone homeostasis [79, 80]. Epithelial cells are the first cell type encountered by oral bacteria that normally produce cytokines and chemokines early during the initiation of periodontal disease. These inflammatory mediators are increased later in the progression of the disease when lymphocytes infiltrate the established and advanced lesion [81].

Macrophage Inflammatory Protein 3 Alpha (MIP-3α) (also known as chemokine ligand 20-CCL20), is a chemokine that is secreted by a broad spectrum of cells and tissues, including lymph nodes, appendix, liver, lungs, and endothelial cells and its production can be induced by IL-1β, TNF- α, INF-γ as well as LPS [82]. CCL20 has been shown as critical marker of innate immune responses against microbial invasion [83, 84]. It has been previously reported, that *P. gingivalis* and *F. nucleatum* are excellent inducers of human CCL20 [85, 86]. In a clinical experiment conducted by Hosokawa, Nakanishi, et al., showed via RT-PCR that periodontally inflamed gingival tissue, expresses MIP-3α mRNA and immunohistochemical staining revealed MIP-3α expression on basal layer of

gingival epithelial cells, microvascular endothelial cells, and dense inflammatory cell areas in periodontal disease sections [87]. MIP-3α-expressing gingival epithelial cells colocalized with gingival-infiltrating T cells, as well as gingiva-homing $CD4^+$ T cells from periodontal disease, suggesting chemotaxis effect of MIP-3α. Consistently, the same authors showed low MIP-3α expression in healthy gingival tissue.

C-C chemokine receptor type 6 (CCR-6), a known receptor of CCL20 [88] is expressed by a variety of cells of the innate and/or adaptive immune system, in particular T-helper 17 (Th17) cells, which exert multiple functions via release of cytokine IL-17A in many chronic inflammatory disorders (e.g., atherosclerosis) [89]. Under inflammatory conditions, epithelial cells are thought to be important sources of CCL20 production [88, 90, 91]. According to Liao F, et al., and Dieu MC, et al., CCR6 is the only known receptor for MIP-3α (CCL20) that is chemotactic for lymphocytes and dendritic cells [92, 93]. Previous studies indicate that CCR6 receptors are expressed on memory T cells and immature dendritic cells, where they are infiltrated, as well as ligand MIP-3α, attaches to memory T cells from the adaptive immune system in periodontal disease tissues [92-95].
CCR-6 is expressed on immature dendritic cells, whereas its expression is lost during their maturation [93]. Furthermore, CCR-6 is present on naïve and memory B cells, but not on germinal center B cells [96] and is also expressed on a small fraction of memory T cells [92]. The expression of CCR-6 has been tightly associated with IL-17 and/or IL-22-producing T cells, referred to as Th17 and Th22 cells [97-101]. Results from experimental animal models have shown that CCR-6

and CCL20 play an important role in the migration of Th17 to inflamed sites *in vivo* [102, 103].

Studies have shown that IL-17A (cytokine IL-17, hallmark of Th17 cells) [104] can stimulate epithelial, endothelial, and fibroblastic cells to produce IL-6, IL-8, and prostaglandin E_2 [105]. It is suggested that IL-17A can induce osteoclastogenesis due to induction of receptor activator of nuclear kappa-B ligand (RANKL) on osteoblastic cells, related to osteoclastic bone resorption activity in periodontal diseases [106, 107]. Similarly, it has been shown that Th17 cells exacerbate inflammatory periodontal disease by activating adjacent cells to produce inflammatory mediators. This in turn generates a positive loop for amplification of the inflammatory reaction that results in more tissue destruction [108]. Early studies implicated IL-23 in driving Th17 differentiation, it is now clear that TGF-β, IL-6, IL-1, and IL-21 are the key cytokines involved in Th17 differentiation, whereas IL-23 is required for Th17 cell expansion, survival, and pathogenicity [109-111].

Chapter 5: Micro RNAs (miRNAs)

According to Millar et al., miRNAs are 21-to 23- nucleotide noncoding RNA molecules that are involved in almost all aspects of eukaryotic life, as well as a regulator for biological processes in cross-coordination and functional integration of complex physiological events [112]. Most miRNAs function as gene expression regulators via silencing mechanisms and mRNA cleavage and post-translation repression through RNA-induced gene silencing complex (RISC) [113] and this silencing mechanism is dependent on the complementary level between miRNA and mRNA target [114]. miRNAs are also involved in up-regulating gene expression [115], meaning that miRNAs can regulate and be regulated by target interactions with mRNA that suggests they play a vital role in physiological interactions and disease processes [116].

Recent studies have shown that miRNAs play roles in the homeostasis of periodontal tissue, stem cell differentiation, osteoblast and osteoclast function as well as serves as a response to mechanical stress in the periodontium [117, 118]. The breakdown of the periodontal connective tissue and surrounding alveolar bone are governed by many factors, including microRNAs (miRNAs) that regulate bone formation, resorption, remodeling, repair, and disease [119]. Despite that evidence, the role of miRNAs in oral health and periodontitis remains unknown. Dysregulation of miRNA expression in tissues is reflected in biofluids, such as serum, saliva and crevicular fluids of the gingiva [120, 121]. Therefore, miRNAs may be used as specific and sensitive biomarkers indicative of many diseases. The involvement of miRNAs in various stages during host response against

bacterial infections is highly dependent on the cellular context, with different cell types responding differently to the same pathogen [122]. Recently, several human subject studies have also compared miRNA expression profiles between healthy and diseased gingival tissues [123-126] or healthy and obese subjects [127]. These profiling studies demonstrated that periodontal disease resulted in a changed miRNA expression pattern in diseased tissues. Changes in miRNA levels affect both primary and secondary immune responses of the host against bacterial infection in periodontal tissue.

At present, the most common oral fluid-based molecular biomarkers for periodontitis fall into three general categories: host derived enzymes and their inhibitors, inflammatory mediators and host response modifiers, and tissue breakdown products [128-131]. Most recently, miRNAs have emerged as a novel class of highly sensitive and specific biomarkers. miRNAs remain stable in extracellular fluids due to their packaging and the thus ideally suited to serve as non-invasive biomarkers for periodontal disease [132-134]. While immune cells and non-immune cells synthesize miRNAs intracellularly, they also actively release miRNAs into extracellular environments, including extracellular fluids [135-137]. The released miRNAs are associated with RNA binding proteins or high-density lipoproteins, or they are enclosed within lipid vesicles. They are highly stable in extracellular fluids [138]. Five miRNAs, including miR-142-3p, miR-146a, miR-155, miR-203, and miR-223, have been proposed as markers for periodontal disease [139]; however miRNAs contributing to oral health remain to be identified.

Our lab has recently identified miR-663a up-regulated in human oral epithelial cells exposed to the oral streptococcal commensal bacterium *S. gordonii.* Interestingly, miR-663a inhibited oral pathogen-induced CCL20 expression. This is relevant given the significant role of CCL20 regulating Th17 responses, which are strongly linked to soft and bone tissue destruction in periodontal disease. Other studies have shown that Hydrogen peroxide can up-regulate miRNAs expression and *S. gordonii* is H_2O_2-producing bacteria [47, 140]. Therefore, we hypothesized that hydrogen peroxide produced by *S. gordonii* modulates miR663a expression and CCL20 production in oral epithelial cells (Figure 1).

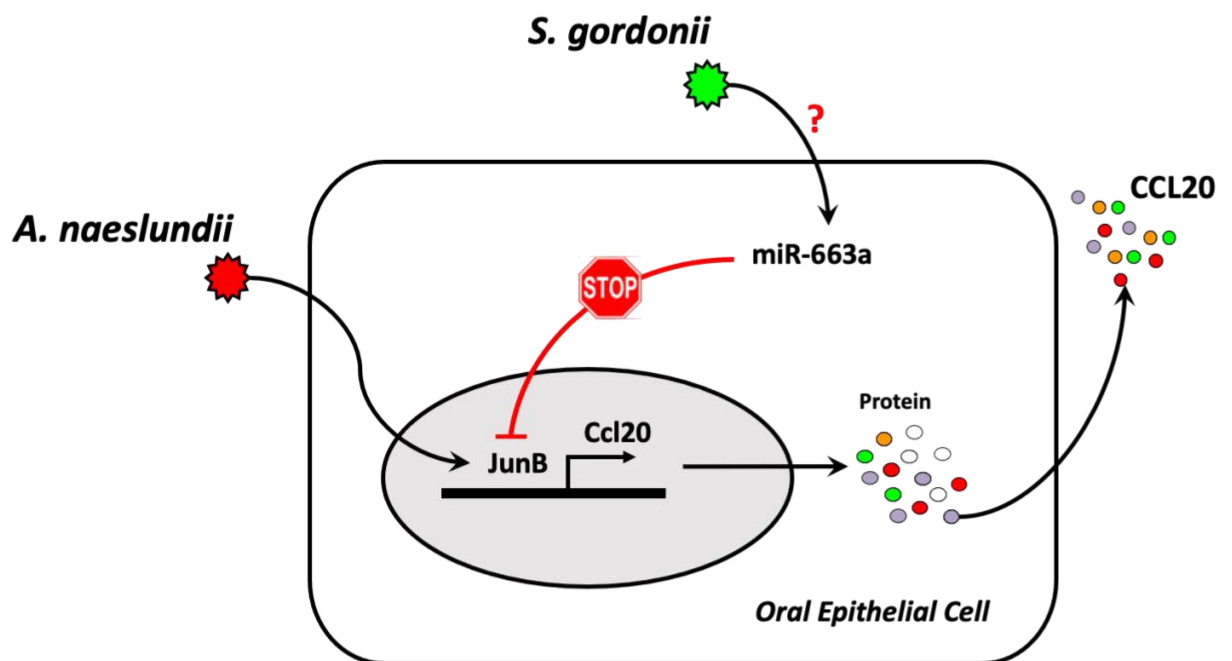


Figure 1 The oral commensal *S. gordonii* (Sg) induces the expression of miR-663a in oral epithelial cells.

MiR-663a reduce oral pro-inflammatory/A. naeslundii-induced CCL20 expression, likely through regulation of the transcription factor JunB. However, the mechanisms through which Sg activates miR-663a expression and regulates CCL20 expression remain unknown.

Chapter 6: Materials and Methods

6.1 Oral Epithelial Cell Culture

The immortalized oral keratinocyte cell line OKF6/hTERT (OKF6) was used and grown in serum-free keratinocyte medium (Ker-SFM, Gibco, Carlsbad, CA) supplemented with 25 µg/mL bovine pituitary extract and 0.2 ng/mL human recombinant epidermal growth factor as previously done (Al-Attar et al., 2018; Dickson et al., 2000). OKF6 cells were incubated at 37°C in 5% Carbon dioxide (CO_2) and cell growth was monitored through an inverted microscope (Nikon Eclipse TI-S, Japan).

To subculture cells, the old medium was removed and OKF6 cells were washed with sterile 1x PBS (Gibco, Canada). After the removal of PBS, 3 mL Trypsin-EDTA (Gibco, Canada) were added to the culture flasks and incubated for 8 minutes at 37°C in 5% CO_2 to detach the cells. Detachment was monitored by inverted microscopy. Further, 6 mL of DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, Canada) were added to inactivate trypsin. Cell suspension was then transferred into a sterile 15 mL tube (Sigma, Darmstadt, Germany) and centrifuged for 6 minutes at 1000 rpm (Sorvall RT6000B Refrigerated Centrifuge). Supernatant was discarded, and cell pellet was re-suspended in Ker-SFM medium with supplements for further cell culture and experiments.

6.2 Bacterial cell cultures

Four streptococcal species abundantly found in the oral microflora were used in these studies: two hydrogen peroxide producers (*Streptococcus gordonii* ATCC 10558 and *Streptococcus sanguinis* ATCC 100556) and two non-hydrogen peroxide producers (*Streptococcus mutans* ATCC 25175 and *Streptococcus salivarius* ATCC BAA-2593). *Actinomyces naeslundii* (ATCC 12104D-5), grown in an anaerobic chamber, was also used as positive control for CCL20 induction in OECs. All bacteria were grown on blood agar plates (BBL, Benton, Dickinson and Company, USA) at 37°C with 5% CO_2 for 24 hours. Then colonies were inoculated in 4mL of brain-heart infusion (BHI) medium (Brain heart infusion, BD; Yeast extract, BD; L-cysteine, Sigma; Hemin, Sigma; Vitamin K; Sigma) and cultured under the same conditions overnight (12-24h) to reach exponential growth. Bacterial growth was monitored by determination of optical density (OD) (Beckman Coulter, DU 530 Life Science UV/Vis Spectrophotometer). Bacteria diluted 1:50 in BHI broth was counted using a microscope (Olympus BX-41, B&B Microscopes Limited) and a hemocytometer, to adjust bacterial cell suspensions for OKF6 cell challenge experiments.

6.3 miR-663a expression analysis by qPCR.

We first evaluated the effect of hydrogen peroxide (H_2O_2) in the expression of miR-663a by OKF6 cells. Cells exposed to *S. gordonii* (1:5) or incubated only with Ker-SFM were used as a positive and negative controls. OKF6 cells ($1x10^5$/well) in 1 mL of Ker-SFM were seeded in 24-well plates overnight. Then, cells were treated with different concentrations of H_2O_2 (0.1, 0.5, and 1mM) (Sigma, Darmstadt, Germany) or infected with *S. gordonii* MOI 1: 5 for 24 hours. Then, supernatants were removed, and miRNA isolation performed using the miRNeasy Mini Kit (Qiagen, Germantown, MD, USA). Briefly, cells were lysed with Trizol (QIAzol) (Qiagen, Germantown, MD, USA) and placed in 1.5 mL tubes, homogenized, and incubated at room temperature for 5 minutes. Chloroform, minimum 99% (Sigma, Darmstadt, Germany) was added to the homogenized mixture and shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes, and then centrifuged at 12,000 x g for 12 minutes at 4°C. The upper aqueous phase was collected, and 1.5 volumes of 100% ethanol (Fisher BioReagents, USA) were added to the mix to further disrupt any bound RNA fragments. The mixture was transferred into a collection tube provided by the miRNeasy kit and centrifuged at 8000 x g for 15 seconds to collect all the RNA from the sample, the waste was removed. Buffer RWT (Qiagen, Germantown, MD, USA) was added to the collection column to purify the miRNA sample and centrifuged for 15 seconds at 8000 x g. Buffer RPE (Qiagen, Germantown, MD, USA) was added to the collection column to wash off membrane-bound RNA and centrifuged twice (for 15 seconds and 2 minutes at 8000 x g) to ensure the

complete removal of total RNA samples from the membrane in the column. Then the mixture was added to a new collection tube and centrifuged at full speed for 1 minute. This mixture was then placed in a 1.5 mL collection tube and 30µL RNAse-free water (Qiagen, Germantown, MD, USA) was added to the membrane directly and centrifuged to elute the samples off the membrane into the flow through liquid. The concentration of total RNA and purity was checked using Thermo Scientific NanoDrop 1000 (Thermo, Waltham, MA). Concentration of total RNA was analyzed using a NanoDrop software and the ratios of A260/A280 of total RNA 1.9-2.0 to ensure good quality. Expression analysis of miR-663a was performed using miRCURY RNA miRNA PCR Starter Kit (Qiagen, Germantown, MD, USA). To prepare the cDNA, the total RNA samples were diluted to 5 ng/µL using RNAse-free water (Qiagen, Germantown, MD, USA). The reverse transcriptase reactions were prepared over ice. The MIX buffer solutions used were 2 µL 5x miRCURY RT Reaction Buffer (Qiagen, Germantown, MD, USA), 4.5 µL RNAse-free water, 1 µL 10x miRCURY RT Enzyme Mix (Qiagen, Germantown, MD, USA), and 0.5 µL UniSp6 RNA spike-in (Qiagen, Germantown, MD, USA) to make a total volume of 8 µL MIX, plus 2 µL Template (sample) volume to equal out to 10 µL. The primary purpose of UniSP6 spike-in and the matching primer is used as an inter-plate calibrator to act as a synthetic control template for the quality, cDNA synthesis and PCR amplification for miRCURY. The samples were then placed in a Thermocycler (Eppendorf Mastercycler, USA) and reverse transcription performed incubating samples for 60 minutes at 42°C, 5 minutes at 95°C to heat

inactivate the reverse transcriptase, and immediately cooled to 4°C. The samples were stored at 4°C.

For the qPCR reaction, the cDNA samples were diluted 1:60 using RNAse-free water. The reaction mixture was then prepared by adding 5 µL 2x miRCURY SYBR Green Master Mix (Qiagen, Germantown, MD, USA), 0.5 µL PCR Primer mix, specific for miRNAs (Qiagen, Germantown, MD, USA), 3 µL cDNA template (Qiagen, Germantown, MD, USA), and 1 µL RNAse-free water per sample. The mixture was then vortexed to homogenize the solution and then 7 µL of Mix and 3 µL of sample were pipetted into a 96-well PCR plate (Qiagen, Germantown, MD, USA). The plate was then centrifuged at room temperature for 2 minutes and then was placed in the thermocycler for analysis (LightCycler480, Roche, Basel, Switzerland). PCR initial heat activation for 2 minutes at 95°C, a two-step cycle of denaturation for 10 seconds at 95°C and combined annealing/extension for 60 seconds at 56°C for 40 independent cycles. The melting curve analysis was between 60 and 95°C. Concentration ratios for the target miRNAs were calculated by normalizing to the endogenous control miR103a-3p, a commonly used miRNA reference, or house-keeping gene will not be affected during the reactions, [141] using the $2^{-\Delta\Delta CT}$ method.

Evaluation of the effect of oral streptococcal species hydrogen peroxide producers or non-producers in miR-663a expression by OKF6 cells were similarly evaluated. Briefly, OKF6 cells were exposed to low (MOI 1:5) or high (MOI 1:50) bacteria concentrations of either H_2O_2-producers (*S. gordonii* and *S. sanguinis*) or

non-H_2O_2-producers (*S. mutans* and *S. salivarius*) for 24h and miR-663a expression evaluated through qPCR as described above.

6.4 Effect of catalase in rescuing A. naeslundii-induced CCL20 expression inhibited by S. gordonii and S. sanguinis.

Protocol for this experiment follows the ELISA MAX^{TM} Deluxe Set Human CCL20 (MIP-3alpha) Protocol (BioLegend, San Diego, CA). OKF6 cells ($1x10^5$ cells/well) in 1 ml Ker-SFM medium with supplements were seeded in a 24-wells plate (SPL Cell culture plate, Korea) overnight. Then, OKF6 cells were exposed to *A. naeslundii* MOI 1:50 in presence of S*. gordonii* or *S. sanguinis (*MOI 1:5)*,* with or without catalase (10 or 100 units) for 24 hours, and supernatant collected and stored in labelled centrifuge tubes on ice. Cells were then washed twice with 1mL ice cold 1x PBS, treated with 300 µL RIPA Buffer with proteinase inhibitor, placed on an orbital shaker over ice for 20 minutes (500 rpm with a 0.3 cm circular orbit), scraped from the 24-well plate and pipetted into a labelled centrifuge tube for centrifugation (cell lysates: 14,000 rpm in 4°C for 15 minutes; supernatants: 5000 rpm at 4°C for 5 minutes). Clear supernatant was collected into a new centrifuge tube and immediately placed in -20°C while the pellet was discarded. Supernatants were normalized to total volume and cell lysates were normalized to total protein. For CCL20 determination, 100 µL diluted Capture Antibody solution was added to each well in a 96 microwell plate (BioLegends) and placed in 4°C overnight. The 96-well microwell plate was then washed 4x using 300 µL Wash Buffer, blocking the plate with 200 µL 1X Assay Diluent A (BioLegends), sealing the plate and incubating at room temperature (RT) on a plate shaker for one hour. After 4 washes with Wash Buffer, 50 µL 1X Assay Diluent A (BioLegends) were added to the wells, followed by addition of 50ul of recombinant CCL20 standards or

supernatant or cell lysate samples. Plate was incubated at RT for 2 hours. Cell lysates were diluted 1:9 with 1X Assay Diluent A (BioLegends) before analysis

After washes, 100 µL Detection Antibody solution (BioLegends) were added into each well and plate incubated at RT for one hour. Finally, 100 µL diluted Avidin-HRP solution (BioLegends) were added to each well, incubated for 30 minutes, washed five times, and 100 µL Substrate Solution F (BioLegends) added to each well. Plate was incubated in the dark for 15 minutes and reaction stopped by adding 100 µL Stop Solution (sulfuric acid, 2N H_2SO_4) to each well. Plate was read with a spectrophotometer (Molecular Devices, SpectraMax M2) at an absorbance at 450 nm and 570 nm. (SoftMax Pro 7 version 7, Data Acquisition and Analysis Software, Molecular Devices).

6.5 Cell viability

OKF6 cells (1x10^5 cells/well) in 1 ml Ker-SFM medium with supplements were seeded in a 24-wells plate (SPL Cell culture plate, Korea) overnight. After different treatments with bacteria, OKF6 cells were monitored using inverted microscope (Nikon). Then, culture media was removed, OKF6 cells washed three times with 1xPBS and trypsinized as described above. Cells were then transferred to 1.5 mL tubes for centrifuging. (Microfuge 20R, Beckman Coulter, Germany). Cell pellets were resuspended in Ker-SFM and counted using trypan blue staining in a 1:1 ratio using Lifestyle Cell Counting (Countess II, Life Technologies). The total concentration and percent (%) of alive cells were recorded using Excel spreadsheet.

6.6 Statistical Analysis

To account for heterogeneity of variance across treatment groups and potential correlation among samples taken from the same experiment, linear mixed models were fit for each outcome variable to test for differences across treatment groups. Random effects were included for wells and adjustments were made to account for differences across experiments, as necessary. In addition, a log transformation was used to help stabilize the variance across treatment groups. For models containing a significant overall fixed effect for treatment, pairwise comparisons and/or subset mixed models were run, as appropriate. Kenward-Roger adjustments were used to correct for negative bias in the standard errors and degrees of freedom calculations induced by small sample sizes. Across all analyses, a *p*-value of less than 0.05 was considered significant. All analyses were completed in SAS 9.4 (SAS Institute Inc.; Cary, NC, USA).

Chapter 7: RESULTS

7.1 Effect of H_2O_2 on miR-663a expression

We first determined if miR-663a expression could be up-regulated by hydrogen peroxide (H_2O_2) in oral epithelial cells (OKF6). All tested H_2O_2 concentrations induced higher miR-663a expression levels when compared with unstimulated cells (Control), although only 0.5 and 1 mM reached significance (**Figure 2**). Expression levels of miR-663a induced by H_2O_2 were about 6-fold higher compared with the effect of *S. gordonii*.

7.2 Effect of catalase in S. gordonii-induced miR-663a expression in oral epithelial cells.

To determine if H_2O_2 produced by *S. gordonii* was involved in miR-663a expression in oral epithelial cells, we exposed OKF6 cells to *S. gordonii (Sg)* [1:5] in presence or absence of the enzyme catalase and miR-663a expression determined as described in methods. As expected, a robust miR-663a expression was observed in cells exposed to *Sg*. Interestingly, this response was attenuated by presence of Catalase, especially at higher concentrations (100U). Similarly, H_2O_2 treatment increased miR-663a expression and catalase significantly reduced this response, which supports appropriate enzymatic activity in the used experimental conditions. (**Figure 3**).

7.3 Effects of oral streptococcal species in miR-663a expression.

Oral streptococci are one of the most abundant species in the oral microbiome. Species that belong to the streptococcal group mitis (*e.g., S. gordonii, S. sanguinis*), are recognized as oral bacteria with the ability to produce higher amounts of hydrogen peroxide as a mechanism of defense to kill or inhibit growth of other bacteria within microbial communities [37, 42-44]. To test the hypothesis that other oral streptococcal species producers of hydrogen peroxide could also enhance miR-663a expression in oral epithelial cells, oral epithelial cells were exposed to selected oral streptococci producers of H_2O_2 (*i.e., S. gordonii* and *S. sanguinis*) and non-H_2O_2 producers (*i.e., S. mutans* and *S. salivarius*). A significant dose-response increase in miR-663a expression was observed in cells exposed to *S. gordonii* and *S. sanguinis* but not to *S. mutans* and *S. salivarius*, when compared with mock unstimulated cells (**Figure 4**).

7.4 Effect of catalase in CCL20 expression inhibited by S. gordonii and S. sanguinis.

Our group recently found that miR-663a induced by *S. gordonii* in oral epithelial cells can reduce CCL-20 secretion induced by oral pro-inflammatory/pathogenic bacteria (*e.g., A. naeslundii and F. nucleatum*). Since the above results show that H_2O_2 producing streptococcal species impact miR-663a expression in OKF-6 cells, we hypothesized that catalase would rescue bacteria-induced CCL20 secretion inhibited by *S. gordonii*. As previously observed by our group, only oral epithelial cells exposed to *A. naeslundii* secreted significant amounts of CCL20 into supernatants but not *S. gordonii* or *S. sanguinis* (**Figure 5A**). Consistently, challenge of oral epithelial cells with *A. naeslundii* in presence of *S. gordonii* or *S. sanguinis* secreted significantly lower amounts of CCL20. Nevertheless, presence of higher concentrations of catalase increased *A. naeslundii*-induced CCL20 levels in supernatants that were inhibited by *S. gordonii.* Similar effect was observed with *S. sanguinis,* although increased CCL20 secreted levels were not significantly different from levels induced by *A. naeslundii* and *S. sanguinis* only in media. (**Figure 4A**). Evaluation of CCL20 levels in cell lysates in response to similar treatments showed comparable results of what was observed with supernatants (**Figure 5B**).

7.5 Cell viability of oral epithelial cells in response to bacterial and hydrogen peroxide treatments.

In general, the oral streptococcal species *S. gordonii*, *S. salivarius* and *S. mutans*, did not significantly affect the cell viability of oral epithelial cells (OECs) at the tested concentrations after 24 h of challenge. Only higher MOI (1:50) of *S. gordonii* and *S. mutans* reduced about 20% cell viability (**Figure 6A**). However, *S. sanguinis* significantly reduced cell viability about 30-50% at MOIs between 1:1 and 1:10. Higher MOI of *S. sanguinis* (1:50) reduced about 60% cell viability of OECs (**Figure 6A**). OECs exposed to H_2O_2 concentrations that enhanced miR-663a expression showed dose-response decreased in cell viability from 25% to 60% at 0.1 and 1 mM respectively, when compared with control unstimulated cells (**Figure 6B**). This range of H_2O_2 concentrations was selected based on preliminary experiments (**Supplementary figure 1**).

FIGURE LEGENDS

Figure 2 Effect of hydrogen peroxide (H2O2) in the expression of miR-663a in oral epithelial cells.

Oral epithelial cells (OKF6) ($1x10^5$/well) were exposed to different concentrations of H_2O_2 for 24h and miR-663a expression determined by qRT-PCR. Cells exposed to S. gordonii [1:5] were used as positive control $^{\&}p \leq 0.05$ bacteria or H_2O_2 vs. Control. The mean ± Standard deviation of three replicates from each treatment condition from two independent experiments is shown.

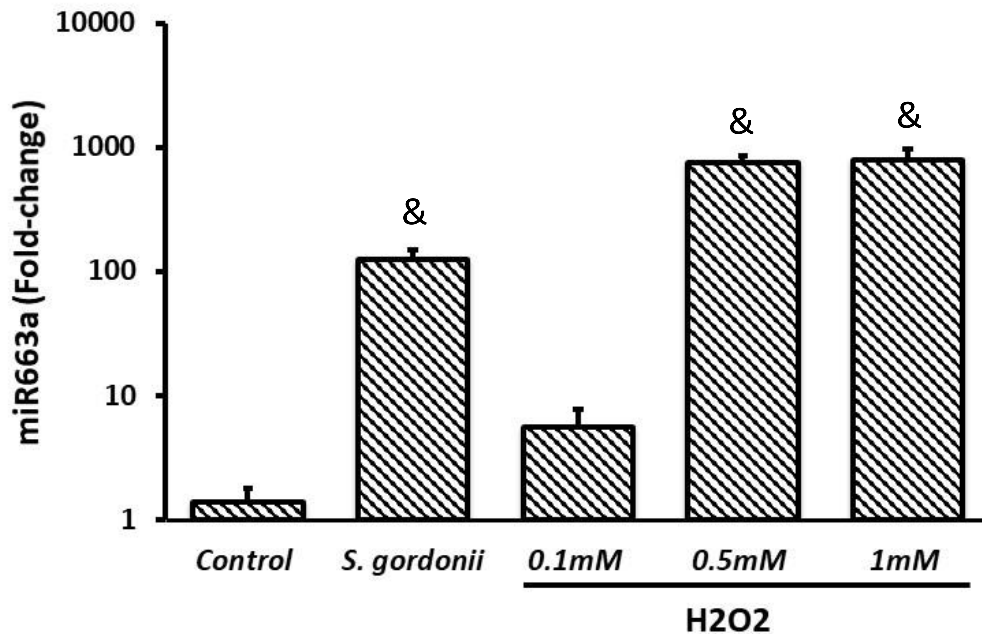


Figure 3 Effect of Catalase in H2O2- and S. gordonii (Sg)-induced miR-663a expression after 24h in oral epithelial cells.

Oral epithelial cells (OKF6) ($1x10^5$/well) were exposed to S. gordonii (Sg) [1:5] or 0.5 mM H_2O_2 for 24h in presence or absence of catalase (10U and 100U), and miR-663a expression determined by qRT-PCR. $^{\&}p\leq0.05$ Sg or H_2O_2 vs. corresponding Mock groups; $^{*}p\leq0.05$ Catalase-treated vs. Media on each group. The mean ± Standard deviation of three replicates from each treatment condition from two independent experiments is shown.

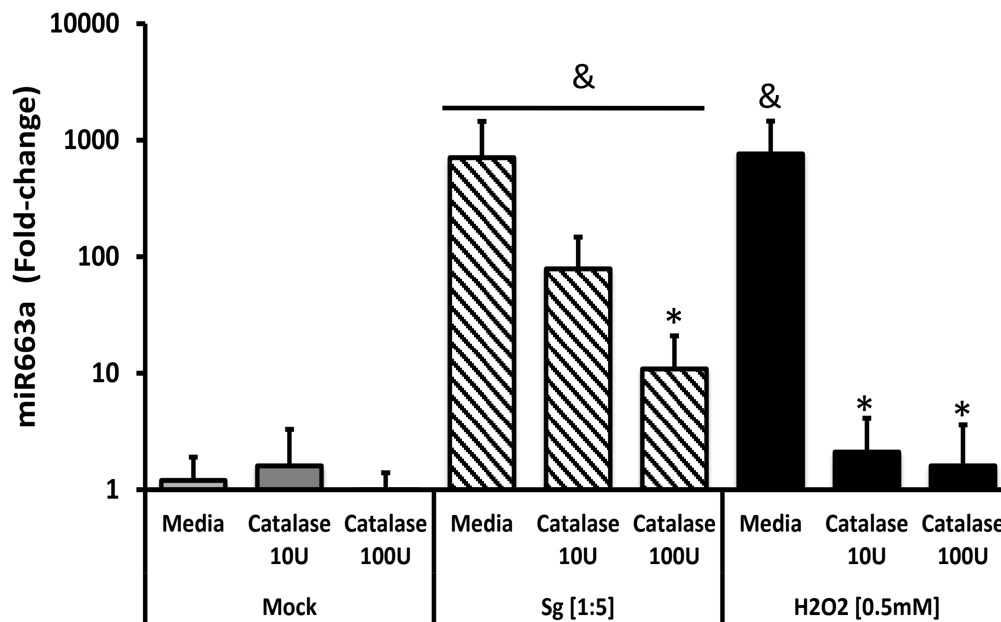


Figure 4 Effect of oral streptococcal species in miR-663a expression.

Oral epithelial cells (OKF6) ($1x10^5$/well) were exposed to oral streptococci H_2O_2 producers (S. gordonii and S. sanguinis) and non-producers (S. mutans and S. salivarius) [MOIs 1:5 and 1:50] for 24h and miR-663a expression determined by qRT-PCR. $^{\&}p \leq 0.05$ bacteria vs. Mock. The mean ± Standard deviation of three replicates from each treatment condition from two independent experiments is shown.

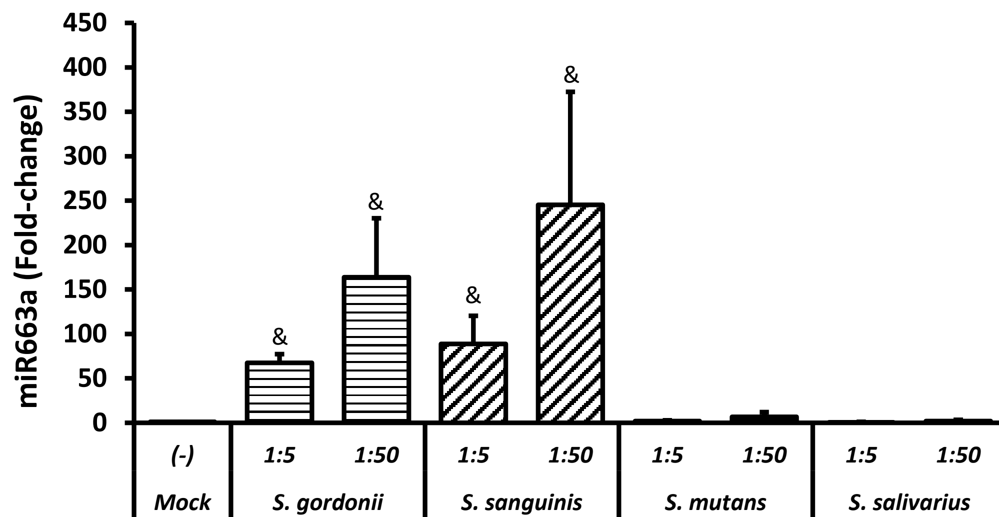
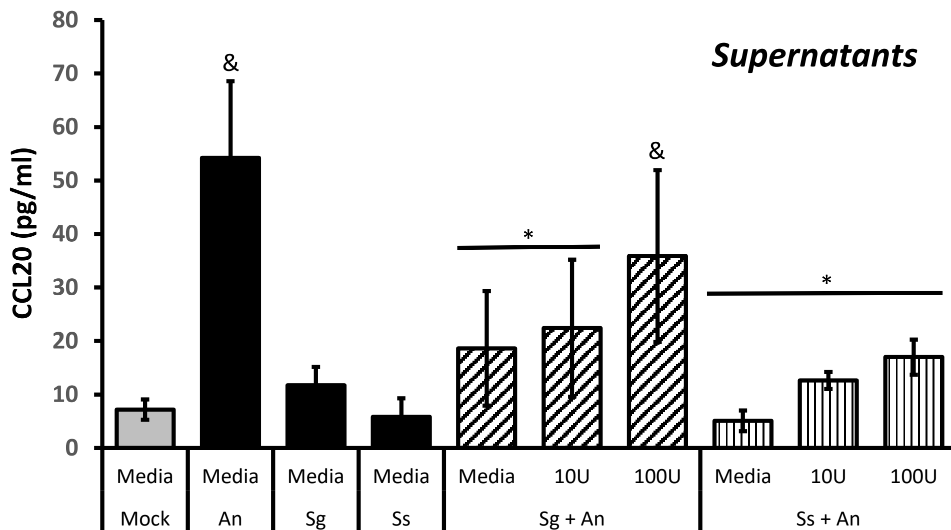


Figure 5 Effect of Catalase in rescuing A. naeslundii (An)-induced CCL20 expression inhibited by S. gordonii and S. sanguinis.

Oral epithelial cells (OKF6) ($1x10^5$/well) were exposed to A. naeslundii (An) [1:50] alone or with S. gordonii (Sg) or S. sanguinis (Ss) [1:5, 1:50] in presence or absence of Catalase (10U or 100U) for 24h and CCL20 levels determined in cell supernatants by ELISA. $^{\&}p \le 0.05$ Media/Mock vs. other treatments. $^{*}p \le 0.05$ Media/An vs Sg+An or Ss+An. The mean ± Standard deviation of three replicates from each treatment condition from two independent experiments is shown.

(A)



(B)

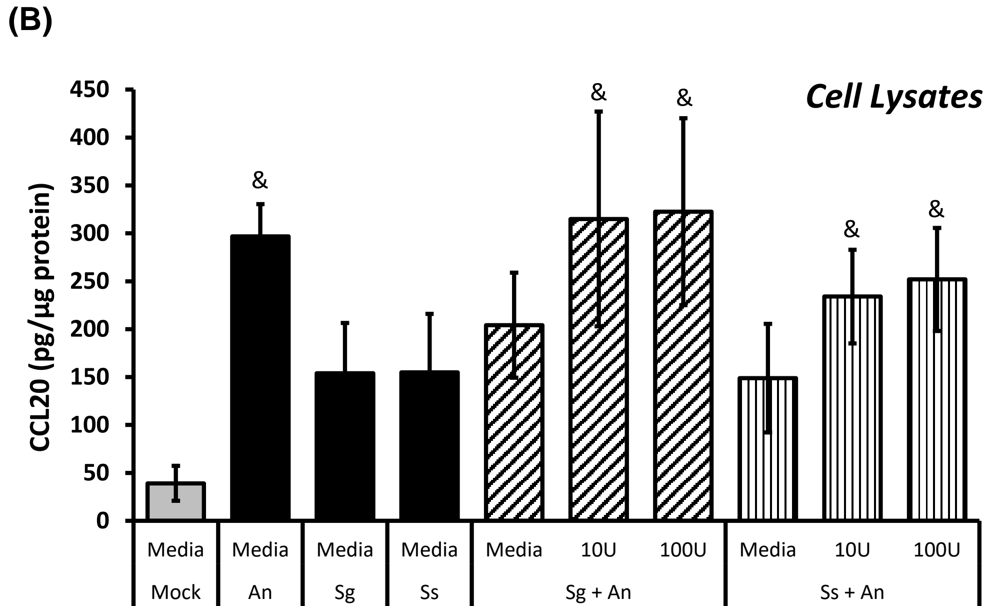


Figure 6 Cell viability of oral epithelial cells exposed to oral bacteria or Hydrogen Peroxide (H2O2).

Oral epithelial cells (OKF6) ($1x10^5$/well) were exposed to **(A)** different MOIs of oral streptococci or **(B)** different concentrations of H_2O_2 for 24 hours. Cell viability was determined through trypan blue exclusion using the Lifestyle Cell Counting (Countess II FL Life Technologies, Thermo Scientific Technologies, USA). The means ± Standard deviations of three replicates from each treatment condition from two independent experiments are shown. *$p \leq 0.05$ bacteria- or H_2O_2-treated groups vs. Control unstimulated cells.

(A)

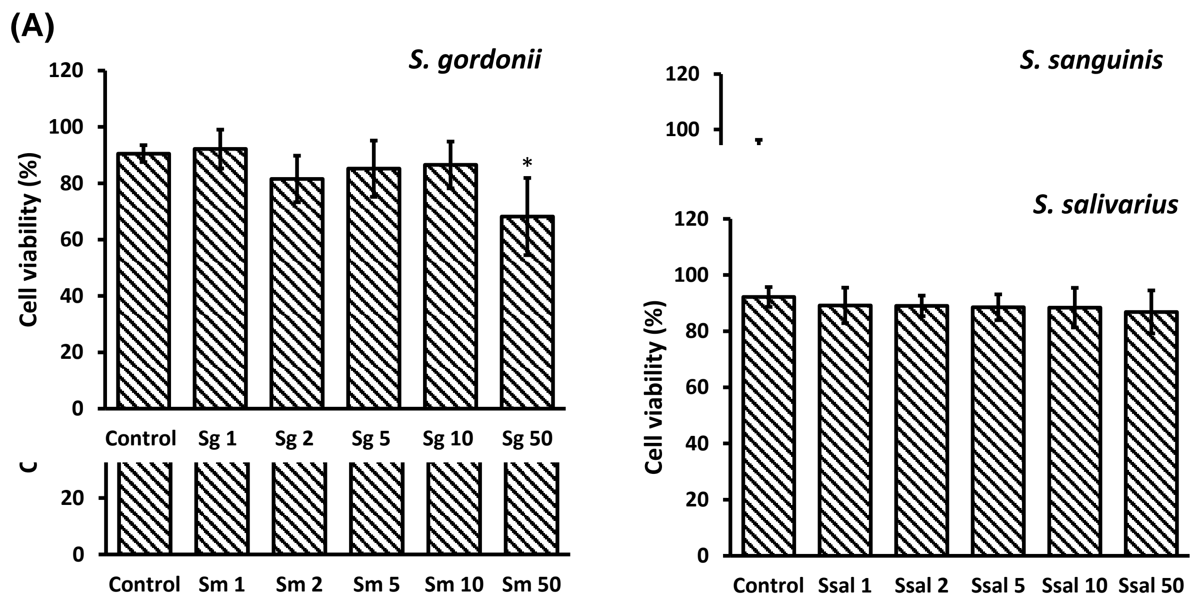
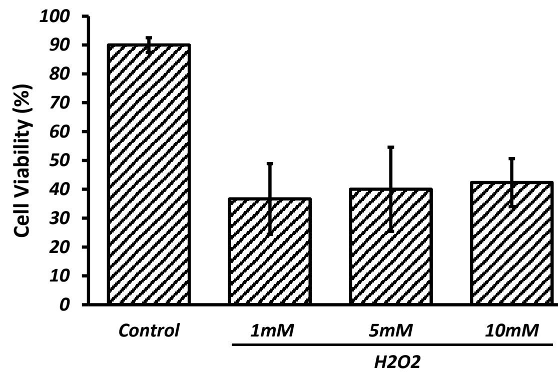


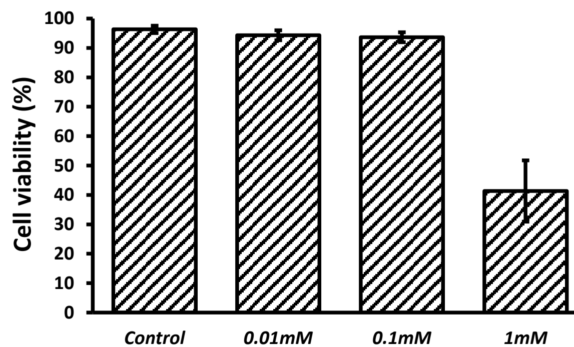
Figure 7 Preliminary experiments of cell viability in oral epithelial cells exposed to Hydrogen Peroxide (H2O2).

Oral epithelial cells (OKF6) were exposed to different concentrations of H_2O_2 for 24 hours. First, a range of H_2O_2 previously tested in vitro with human oral cells by other groups was evaluated [1-10 mM]. In a second experiment an adjusted range of H_2O_2 concentrations was evaluated showing that any effect in OEC viability could be between 0.1 and 1 mM. Cell viability was determined through trypan blue exclusion using the Lifestyle Cell Counting (Countess II FL Life Technologies, Thermo Scientific Technologies, USA). The means ± Standard deviations of three replicates from each treatment condition are shown.

Experiment 1.



Experiment 2.



Chapter 8: DISCUSSION AND CONCLUSIONS

The mechanisms by which commensal bacteria regulates oral pathologic inflammation (*e.g.,* periodontitis) remain unknown. A better understanding of these bacteria-associated anti-inflammatory mechanisms could provide future opportunities to prevent periodontal disease. Our lab has recently found that the oral commensal bacteria *S. gordonii* up-regulates the expression of miR-663a in oral epithelial cells (OECs), and this miRNA was able to decrease oral pathogen-induced CCL20 expression. This is relevant given the significant role of CCL20 regulating T-helper 17 (Th17) and T-regulatory (Treg) cell responses at mucosal surfaces [87, 92, 142, 143], which are involved in the pathogenesis of soft and bone destruction occurring in periodontal disease.

Based on the above findings, the ability of *S. gordonii* to produce hydrogen peroxide (H_2O_2), and previous studies showing that H_2O_2 stimulates miRNA expression [144], the central question of this study was whether H_2O_2 produced by *S. gordonii* was involved in miR-663a and CCL20 expression in oral epithelial cells (OECs). To address this question, we tested *in vitro* in human OECs (OKF6) the effect of H_2O_2 as well as H_2O_2-producer or non-producer oral streptococcal species in the expression of miR-663a and CCL20.

Initial treatment of OECs with H_2O_2 enhanced expression of miR-663a, similarly to the effect of *S. gordonii*. This response was about 8-9 times higher in cells exposed to H_2O_2 compared to *S. gordonii* treatment. These findings are consistent with previous studies showing the ability of H_2O_2 to stimulate the expression of specific miRNAs in other cell types (e.g., vascular smooth muscle

cell), although using significantly higher H_2O_2 concentrations between 10-200 μM [144]. Biologically relevant H_2O_2 concentrations that bacteria normally encounter are usually in the nanomolar to lower millimolar range, from 0.51 mmol/L up to 7 mmol/L reported for streptococci although those measurements were mostly performed in batch cultures that may not be reflective of the normal H_2O_2 capacity of streptococcal biofilms [145-148], like the concentrations evaluated in this study (*i.e.,* 0.1-1 mM), which could be more biologically relevant for specific host cell responses.

Accurate measures of H_2O_2 produced by bacteria are challenging. Liu, et. al., quantified the concentration of H_2O_2 produced by *S. gordonii* in the presence of glucose using scanning electrochemical microscopy (SECM), a technique that can scan over a substrate in the *x-y* direction and thus is able to record a unique spatial concentration profile over a surface and has been used in other biological systems to measure the local concentration over soft biological samples and for imaging [149-151]. They detected ranges 0.4 mM-1.6 mM H_2O_2, depending on the distance of the sensing tip from the biofilm, and time duration, because it takes a much longer time to build up detectable H_2O_2 concentrations.

To determine whether H_2O_2 produced by *S. gordonii* is involved in miR-663a expression in OECs, we exposed cells to *S. gordonii* either in presence or absence of the enzyme catalase. Catalase is a highly efficient enzyme that is widely expressed in peroxisomes, decomposes hydrogen peroxide and also inhibits the *SpxB* gene expressing the oxidant formation [50, 152]. As expected, presence of catalase significantly decreased *S. gordonii*-induced miR-663a expression in a

dose-response manner. However, even higher catalase concentrations did not completely abrogate miR-663a expression. This suggests that *S. gordonii*-produced H_2O_2 is involved in up-regulation of miR-663a in OECs. Likely, the amounts of H_2O_2 produced by *S. gordonii* under these experimental conditions could be higher than 0.5 mM given that similar amounts of H_2O_2 were completely abrogated by the same catalase concentrations. The mechanisms by which H_2O_2 could be enhancing miR-663a expression in OECs remain to be determined. Perhaps, the use of a more specific method to analyze the role of H_2O_2 produced by *S. gordonii* in miR663a expression such as a *SpxB* knockout strain would allow to determine if indeed, H_2O_2 or some other factors produced by *S. gordonii could* also be contributing to this response.

H_2O_2 can serve as messenger to carry a redox signal from the site of its generation to a target site and is considered most suitable for redox signaling [60, 153]. H_2O_2 also modulates the activity of transcription factors (e.g., NF-κB, NOTCH, TP53) in mammalian cells [153]. Different patterns of p53-regulated gene expression are initiated in response to different levels of H_2O_2. We have found that miR-663a targets the transcription factor JunB in OECs, which has also been shown to regulate chemokine expression. Thus, the potential activation of JunB by H_2O_2 could be regulated through miR-663a. For example, antioxidants are induced in response to low levels of H_2O_2, where they lower ROS levels and hence, protect the cell from DNA damage, while higher levels also stimulate the expression of pro-oxidants involved in apoptosis [57]. The potential role of these cell signaling pathways in miR-663a expression by OECs needs to be explored in future studies.

Streptococci are one of the most abundant oral microbiome bacterial species normally colonizing the oral cavity. Among these, bacterial species from the mitis group such as *S. gordonii*, are strong producers of H_2O_2 [48]. Thus, other bacterial species from the *S. mitis* group would be able to enhance miR663a expression; nevertheless, streptococcal species from other groups (*e.g.,* group mutans or group salivarius) would fail to do it. Consistent with this hypothesis, only *S. gordonii* and *S. sanguinis* from mitis group were able to increase miR-663a in OECs in comparison to *S. mutans* and *S. salivarius*, which did not stimulate miR-663 expression. The ability of some oral streptococci to produce H_2O_2 has been associated with bacterial killing or regulating the growth of other bacterial species within the biofilms [53]. For example, in the oral biofilm, naturally produced H_2O_2 crosses the bacterial membrane of producer cells, containing pyruvate oxidase (*spxB)* gene, and is released into the environment where it can influence neighboring cells and ultimately oral biofilm ecology [53]. *SpxB*-positive bacterial species are expected to play a particularly important role in early biofilm development, as streptococci comprise significant fractions of newly formed oral biofilms and these biofilms contain significantly higher oxygen concentrations compared with older, mature biofilms [154, 155]. In addition to their antibacterial properties, this group of oral bacteria could also be specifically stimulating the expression of miRNAs such as miR-663a to modulate host epithelial inflammatory responses.

Although, several studies suggest that H_2O_2 can influence signaling pathways that induce some proinflammatory responses [58-60], most recently, it

has been demonstrated that beyond the conventional view of reactive oxygen species as drivers of inflammation, H_2O_2 plays a significant role in host modulation by inhibiting inflammasome responses at respiratory mucosal surfaces [68]. Thus, specific H_2O_2 levels produced by oral streptococcal species could be regulating pathologic oral inflammatory responses triggered by pathogenic/pro-inflammatory bacteria. [156]. The effect of H_2O_2 in host cells seems to be concentration-dependent. For example, hydrogen peroxide has concentration-dependent effects in the conjugation of mammalian proteins to the SUMO ubiquitin-like modifier. SUMOylation is an important regulator of the localization, activity, and stability of many proteins, and thus the concentration-dependent regulation of this protein modification may be important to ensure the appropriate biological response to increasing levels of H_2O_2 [50]. Concentrations of H_2O_2 such as those generated by activated macrophages inhibit conjugation of SUMO and hence reduce SUMOylation.Higher H_2O_2 concentrations (e.g., 100 mM) inhibited conjugation of SUMO, causing an increase in the levels of SUMOylation [157-160].

Consistent with the above hypothesis, ongoing experiments in our lab showed that *S. gordonii* challenge or transfection of OECs with miR-663a downregulated CCL20 expression stimulated by *A. naeslundii.* Given that expression of miR663a was induced by H_2O_2 in OECs and catalase abrogated that effect, we hypothesized that H_2O_2 produced by *S. gordonii* was involved in their ability to reduce *A. naeslundii*-induced CCL20. As expected, decrease in *A. naeslundii*-stimulated CCL20 secretion into OEC supernatants induced by *S.*

gordonii, was rescued by the presence of catalase. Whether rescue of *A. naeslundii*-induced CCL20 blocked by *S. gordonii* with catalase involves down-regulation of miR-663a needs to be determined; however, we found that *S. gordonii*-induced miR-663a expression was significantly decreased by catalase. Of note, similar responses were observed with *S. sanguinis*, another oral H_2O_2 producer. Thus, it is possible that oral streptococci H_2O_2 producers such as *S. gordonii* and *S. sanguinis* could be regulating CCL20 expression induced by oral pathogenic bacteria in OECs through miRNAs such as miR663a. This is an important observation, since increased levels of CCL20/MIP3α in gingival epithelial cells and microvascular endothelial cells as well as a massive infiltration of CCR6-expressing cells have been associated with periodontal disease [87]. Therefore, presence of streptococci species producing H_2O_2 as part of the normal oral microbiome in a symbiotic environment would be critical to maintain homeostasis and oral health. In contrast, reduction in the abundance of these symbionts, during oral dysbiosis induced by environmental (e.g., smoking), host (e.g., Immunodeficiencies), or oral pathogens (e.g., *P. gingivalis*), could be contributing to enhance oral inflammation through variation in the levels of specific chemokines such as CCL20. Similar CCL20 responses were observed in oral epithelial cell lysates, which suggests that regulation of the expression of this chemokine could involve miR-663a-mediated transcriptional and/or post-transcriptional mechanisms, but not CCL20 secretion alterations.

Concentrations of H_2O_2 and *S. sanguinis* with the ability to increase miR-663a expression, reduced about 40% the cell viability of OECs. The potential of

miR-663a to regulate cell death and proliferation have been reported in several cancers (e.g., breast, renal carcinoma) with different outcomes, either increasing or decreasing cell death and proliferation, depending on the type of cancer [161, 162]. Future studies investigating the role of miR663a in OEC apoptosis are warranted.

A body of evidence supports that reactive oxygen species (ROS) such as H_2O_2 are involved in the pathogenesis of several inflammatory diseases such as atherosclerosis, hypertension, diabetic complications, etc., by modulating the expression of genes associated with cell differentiation, proliferation, migration, and apoptosis [163-165]. However, emerging evidence indicates that specific levels of H_2O_2 contribute to regulate bacterial growth of oral pathogens normally colonizing mucosal surfaces in low numbers and reducing inflammation [166-169]. The mechanisms through which oral commensal bacteria prevent pathologic oral inflammatory responses remain unknown. In this study, we have identified a new H_2O_2-mediated regulatory mechanism through which oral streptococcal species could be attenuating host CCL20-mediated pro-inflammatory epithelial responses through miRNAs, thus contributing to maintain an oral and periodontal symbiotic environment. Future studies using organotypic cultures and biofilm models as well as *in vivo* mice models would be needed to validate the functional effect of miR663a in modulating the recruitment of Th17 and Treg cells by oral pathogens and soft and bone tissue destruction, both hallmarks of periodontal disease.

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