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*PEPTOANAEROBACTER STOMATIS* INTERACTIONS WITH NEUTROPHILS

By

Emeri Jimenez Flores

A Thesis

Submitted to the Faculty of the  
School of Dentistry of the University of Louisville

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

In Oral Biology

Department of Oral Immunology and Infectious Diseases

School of Dentistry University of Louisville

Louisville, Kentucky

August 2016

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*PEPTOANAEROBACTER STOMATIS* INTERACTIONS WITH NEUTROPHILS

By

Emeri Jimenez Flores

A Thesis Approved on

August 1, 2016

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## DEDICATION

To my parents Dr. Javier E. Jiménez Gallegos and Dr. Criselda Flores  
Zuniga who never stop believing in me.

## ACKNOWLEDGEMENTS

First and foremost, I want to thank my family, who has been my source of encouragement and inspiration throughout my life. Thanks for the sacrifices you have always made to provide me with opportunities to chase my dreams.

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## ABSTRACT

### *PEPTOANAEROBACTER STOMATIS* INTERACTIONS WITH NEUTROPHILS

Emeri Jimenez Flores

August 1, 2016

*Peptoanaerobacter stomatis* is a newly appreciated taxon, present in high numbers in oral biofilms from periodontal patients. However, little is known about this organism's potential virulence and its interaction with the host immune response. Therefore, to better understand the role of *P. stomatis* in periodontitis, we studied its interactions with neutrophils.

Human neutrophils were challenged with *P. stomatis* strain CM2 (multiplicity of infection 10), or *S. aureus* AF488 (positive control) for 30 min. Bacterial internalization was examined by ImageStreamX imaging flow cytometry. Bacterial viability was assessed by colony-forming unit assays and BacLight assays. Phagocytosis-stimulated respiratory burst response was measured by flow cytometry. The fusion of specific and azurophil granules with bacteria-containing phagosomes was tested by immunostaining using confocal microscopy. Neutrophil granule exocytosis was measured by ELISA and flow cytometry.



After 30 min of incubation, 20% of neutrophils were infected with *P. stomatis*. Approximately, 43 % of the bacterial inoculum was killed within a 30 min interaction with neutrophils. *P. stomatis* induced a higher respiratory burst response compared to *S. aureus*. When neutrophils were challenged with *S. aureus*, 64% of the bacteria-containing phagosomes were enriched for the specific granule marker, lactoferrin; similarly 52% of *P. stomatis*-containing phagosomes were enriched with the granule marker. Likewise, 82% of *P. stomatis*-containing phagosomes were enriched with the azurophil granule marker, elastase; while 89% of the phagosomes containing *S. aureus* were enriched for this granule marker. *P. stomatis* challenge stimulated the degranulation of the four neutrophil granule subtypes.

These results suggest that although neutrophils had a low phagocytic efficiency for *P. stomatis*; this periodontal pathogen was able to trigger the neutrophil killing mechanisms contributing to promote the chronic inflammation and tissue destruction that characterize periodontal diseases. These data demonstrate previously unexplored aspects of this newly appreciated taxon and how these organisms modulate neutrophil function.

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## CHAPTER 1

### INTRODUCTION

#### Periodontal Disease

Periodontal diseases are one of the most prevalent infectious diseases worldwide, affecting almost half of the U.S. population (Eke et al., 2015). These disorders affect one or several of the tissues supporting the teeth, which are the alveolar bone, periodontal ligament, cementum and gingiva; and may have inflammatory, traumatic, genetic or metabolic etiology (Pihlstrom et al., 2005). Nonetheless, the term periodontal disease usually refers to the most common inflammatory disorders, gingivitis and periodontitis, which are both caused by pathogenic microbiota in the biofilm that accumulates on the teeth surfaces on a daily basis (Pihlstrom et al., 2005).

Gingivitis, the mildest form of periodontal disease, is highly prevalent and readily reversible by simple, effective oral hygiene. On the other hand, chronic

inflammatory periodontal disease (CIPD), also known as periodontitis, is a chronic inflammation of the tooth-supporting tissues (periodontium) that causes loss of connective tissue and alveolar bone (Darveau, 2010; Sosroseno & Herminajeng, 1995). Notably, chronic periodontitis is the most common cause of tooth loss worldwide, and a recent epidemiologic study estimated that 47 percent of American adults suffer from periodontitis (Eke et al., 2015; Friesen et al., 2014).

Chronic periodontitis is considered a silent disease since symptoms may not appear until its advanced stages. Patients with advanced periodontitis often present with occasional pain and discomfort, impaired mastication, halitosis, gum recession, periodontal abscesses, loose or separating teeth and eventually tooth loss (Pihlstrom et al., 2005). The diagnosis of periodontitis is based on results of clinical and radiographic examinations, evaluating factors such as gingival bleeding on probing, clinical attachment levels, tooth mobility, furcation involvement and radiographic evidence of bone loss (Kaur et al., 2013). Both preventive and treatment measures are aimed at removing and controlling the periodontal biofilm; they include scaling, root planning and curettage (Pihlstrom et al., 2005). The complementary use of antiseptic drugs and/or antibiotics can potentially contribute to the treatment outcome. Unfortunately, these treatments are only a temporary attempt to arrest disease progression, since infection almost always returns (Douglass, 2006). Moreover, even in cases where the inflammation is successfully resolved and some tissue is regenerated, a complete restoration of the lost tooth support is impossible (Pihlstrom et al.,

2005). Additionally, periodontitis can adversely affect systemic health; it has been associated with increased risk for rheumatoid arthritis, cardiovascular disease, diabetes and with adverse pregnancy outcomes (Davé & Van Dyke, 2008; Kaur et al., 2013; Shub et al., 2006).

Periodontitis results from the complex interaction of diverse etiologic components, such as the biofilm (also called dental plaque), the host's genetic traits, as well as environmental, social, and behavioral aspects (Hernández et al., 2011; Pihlstrom et al., 2005). Due to its permanent proximity to periodontal tissues, dental plaque causes a continuous challenge to the host's innate immune system, resulting in an incessant inflammatory response (Darveau, 2010; Hernández et al., 2011). In healthy periodontal sites, the host response resists colonization by periopathogens while avoiding collateral damage to surrounding tissues. Contrastingly, the inflammatory response that develops towards the chronic presence of pathogens in periodontal biofilms is more robust and dysregulated, resulting in a non-resolving inflammatory response that induces periodontal tissue destruction (Costalonga & Herzberg, 2014; Hajishengallis, 2014; Uriarte et al., 2016).

## Oral microorganisms and periodontal diseases

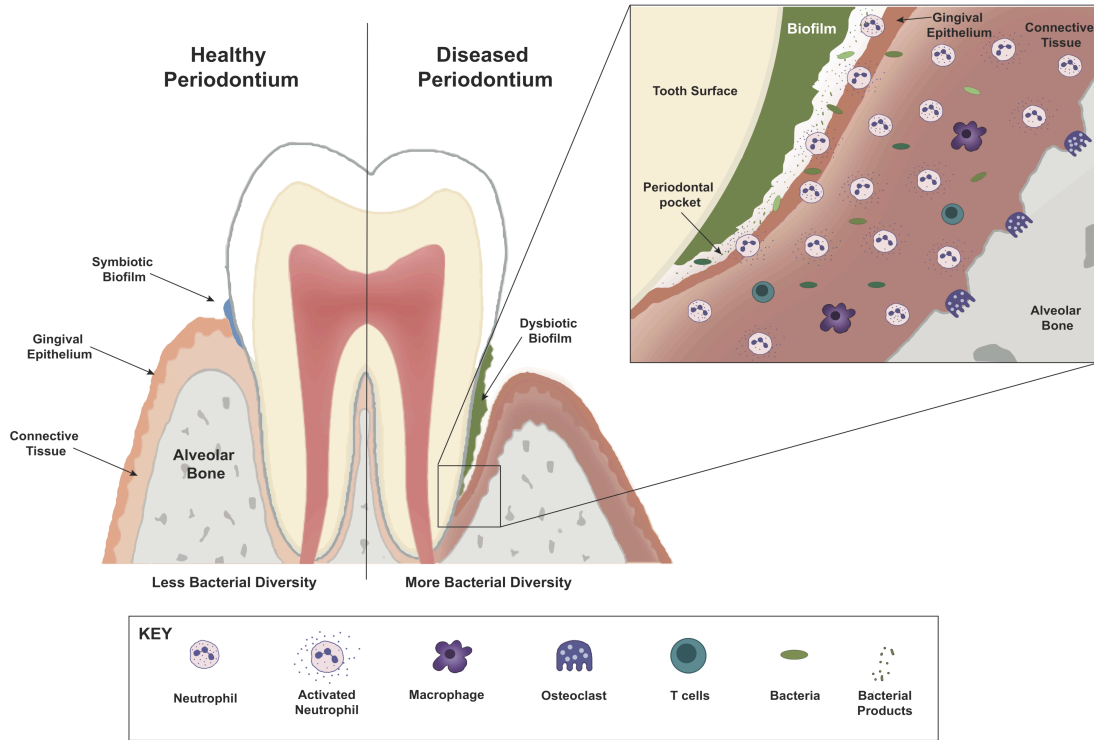
It was originally speculated that the oral microbiota contained more than 500 hundred species of aerobic and anaerobic bacteria that live in harmony with their healthy host. Yet recent improvements in sequencing technology have revealed an even more diverse bacterial microbiota and suggest that a large proportion of the already recognized organisms remains uncharacterized (Griffen et al., 2011; Pihlstrom et al., 2005; Wade, 2013).

For years, periodontal disease studies have focused on three organisms (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) considered to be keystones for the onset of periodontitis (Hajishengallis & Lamont, 2012). However, due to the development of culture-independent techniques, such as 16sRNA sequencing other bacterial species have been identified from periodontal biofilms (Kistler et al., 2015). These observations have raised questions about the role other pathogens may play in the disease process.

Current theories on the etiology of periodontitis favor the idea that the disease develops because of a shift in the composition of the microbial community from symbiosis to dysbiosis (Fig.1) (Uriarte et al., 2016). It has been suggested that the decrease in beneficial symbionts might be caused by several risk factors, such as smoking, an immunocompromised host, tissue injury, and/or



the colonization of the oral cavity by organisms with enhanced pathogenic potential (Berezow & Darveau, 2011; Griffen et al., 2011; Hajishengallis & Lamont, 2012; Hajishengallis & Lamont, 2016; Lamont & Hajishengallis, 2015; Uriarte et al., 2016). This current model describing the etiology and pathogenesis of periodontitis is called the poly- microbial synergy and dysbiosis (PSD) model. It suggests that a discrepancy between commensal and pathogenic bacteria results in a recurring inflammatory state that clears beneficial bacteria while facilitating the growth of periodontal pathogens (Hajishengallis, 2014; Hajishengallis & Lamont, 2012). Further facilitating disease progression, dysbiotic microbial communities exhibit synergistic interactions for enhanced colonization, nutrient procurement, and persistence in an inflammatory environment that promotes their adaptive fitness and disease progression (Hajishengallis, 2014). Pathobionts are organisms that reside in the healthy oral microbiota, but can take advantage and proliferate on inflammation sites. They manipulate the host immunity and propagate inflammation, enhancing the environment for other periopathogens (Hajishengallis, 2014; Hajishengallis, 2015).



**Figure 1. A schematic of the host response in a healthy and diseased periodontium**

The local oral microbiota of a healthy periodontal site (left) is characterized by low bacterial diversity and quantity. In this homeostatic scenario, the symbiotic biofilm enhances the recruitment of neutrophils, which mount a controlled acute inflammatory response. However, in the case of periodontitis (right) genetic predisposing conditions of the host, and/or environmental risk factors favor the colonization of exogenous pathogens, which results in an increase in bacterial diversity, formation and overgrowth of a dysbiotic bacterial biofilm, with massive recruitment and neutrophil infiltration invading the gingival epithelium, and the crevicular fluid. The result of this chronic inflammation involves both the innate and adaptive immune cells which results in periodontal lesions (right, and inset) with connective tissue and alveolar bone destruction (Uriarte et al., 2016).

In order to cause periodontitis, periodontal pathogens require different virulence factors that allow them to colonize the periodontium, protect themselves from the host response and promote tissue damage (Socransky & Haffajee, 1991). For example, to counteract the immune response some bacteria mimic host antigens, secrete proteases, or release leukotoxins (like *Aggregatibacter actinomycetemcomitans*) (Pihlstrom et al., 2005; Socransky & Haffajee, 1991). *T. forsythia* mitigates bacterial clearance by using a serine protease inhibitor (Miropin) to suppress neutrophil proteases such as elastase and cathepsin G, and secretes metalloproteinases to evade the innate immune response (Ksiazek et al., 2015; Uriarte et al., 2016). The major outer sheath protein (Msp) of *T. denticola* contributes to disease progression by modulating neutrophil signaling pathways involved in chemotaxis and phagocytosis (Amin et al., 2004; Batista da Silva et al., 2004; Magalhães et al., 2008; Uriarte et al., 2016). Moreover, *P. gingivalis* has several virulence factors such as lipopolysaccharide (LPS), proteolytic enzymes, and a capsule that allow it to modulate the immune response mechanisms and promote tissue damage (Potempa & Pike, 2009; Uriarte et al., 2016).

## Peptoanaerobacter stomatis and periodontal diseases

*Peptoanaerobacter stomatis* is a newly appreciated pathogen that has been recognized to be present in oral biofilms in patients with periodontal diseases (Kumar et al., 2005; Murphy & Frick, 2013; Sizova et al., 2015). It was proposed by Sizova et al. (2015) as a novel genus and species within the family of *Peptostreptococcaceae*, and its bacterial strains ACC19a, CM2, CM5, and OBRC8 represent the first known cultivable members of the human oral taxon 081.

*P. stomatis* is a member of the oral commensal flora and probably accounts for isolates that were previously thought to be *P. anaerobius*, as it is now hypothesized that *P. anaerobius* is not, in fact, an oral commensal (Murphy & Frick, 2013). Downes & Wade (2006) suggested that the novel species, *P. stomatis*, originates from the oral cavity in contrast to *P. anaerobius*, which is involved in infections below the waistline.

The *P. stomatis* genome is 2,541,543-bp long and contains 2,277 protein-coding and 54 RNA genes; it has a 30.37 mol % G + C content (Sizova et al., 2015). Based on strains isolated from the human oral cavity, *P. stomatis* cells can be described as Gram-positive obligate anaerobic rods, about 1.2 – 2.5 µm long and 0.4 – 0.8 µm wide, often occurring in pairs or short chains (Sizova et al., 2015). Thanks to its peritrichous flagella *P. stomatis* is highly motile (Downes & Wade, 2006). *P. stomatis* is mildly saccharolytic and ferments glucose,

maltose, and sucrose weakly. Its major metabolic end products from glucose fermentation are acetate and propionate (Downes & Wade, 2006; Sizova et al., 2015).

*P. stomatis* colonies are approximately 0.5 mm in diameter and are non-hemolytic, circular, convex and beige in color (Murphy & Frick, 2013; Sizova et al., 2015). Moderate growth is obtained in broth media at a temperature range of 30–42°C and is further enhanced by the addition of fermentable carbohydrates. Yeast extract is required for growth on glucose, sucrose, and maltose (Downes & Wade, 2006; Murphy & Frick, 2013; Sizova et al., 2015).

*P. stomatis* has been associated with infections of the human oral cavity, such as dentoalveolar abscesses, endodontic infections and periodontal disease (Downes & Wade, 2006). Furthermore, oral infections previously attributed to *P. anaerobius* were probably incorrectly identified and were in fact caused by *P. stomatis* (Rôças & Siqueira, 2008). However, further research about this pathogen's virulence factors and its interaction with the host immune response is still needed. To this end, studying the interactions between the innate immune system and *P. stomatis* will provide valuable information to understand the role of this pathogen in periodontal diseases and enhance our understanding of bacterial strategies to subvert innate immunity.

## Neutrophils and Periodontal Disease

Neutrophils, also known as polymorphonuclear leukocytes or granulocytes, are the most prevalent white cell in humans (Quinn, 2014). In the innate immune response, neutrophils are considered the first and most abundant cell type encountered at the site of infection and are responsible for the death and phagocytosis mediated clearance of invaders (Ryder, 2010; Scott & Krauss, 2012); thus, immune evasion is an important mechanism in pathogen survival.

Neutrophils are among the shortest-lived human cells, with a typical circulating half-life of 6–8 h. During infection, the neutrophil lifespan is extended and they are rapidly recruited to the site of infection (Quinn, 2014). As a major component of the innate host response, neutrophils play a key role in maintaining oral health by protecting the tissue against bacterial infection (Ryder, 2010; Scott & Krauss, 2012).

Approximately 30,000 neutrophils transit through periodontal tissue every minute, forming a protective wall between the oral community colonizing the tooth and the periodontium (Darveau, 2010; Uriarte et al., 2016). Their role in periodontal diseases is shown in the increased prevalence of periodontitis in patients with leukocyte adhesion deficiency and neutropenia (Darveau, 2010; Nordenfelt & Tapper, 2011).

The massive accumulation of neutrophils in the periodontal pocket is characteristic of periodontal disease. This hyperactive response causes

disruption of the periodontal tissue homeostasis leading to clinical signs of periodontitis (Hajishengallis, 2014).

Neutrophils have multiple killing mechanisms to eliminate both intracellular as well as extracellular microorganisms (Kolaczkowska & Kubes, 2013). The neutrophils first attempt to clear intruders is by phagocytosis, a receptor-mediated process during which a solid particle is engulfed by the cell membrane into a vesicle called the phagosome. This mechanism depends on the interaction between the neutrophil and the particle or microorganism, and is usually opsonin-mediated or through recognition of pathogen associated molecular patterns (PAMPs) (Amulic et al., 2012). After phagocytosis, neutrophils employ different killing mechanisms in an attempt to destroy infectious agents (Quinn, 2014).

One of the most critical functional mechanisms neutrophils use to attack phagocytized foreign particles is to generate reactive oxygen species (ROS) within the phagosome, such as superoxide ion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hypochlorous acid (HOCl) (Nordenfelt & Tapper, 2011). The generation of ROS is highly damaging to macromolecules such as protein and DNA, therefore resulting strongly bactericidal. Reactive oxygen species may also cause destruction in surrounding tissues, and are capable of inducing immune cell apoptosis (Parker et al., 2014).

During phagocytosis or upon interaction with inflammatory mediators,

neutrophils become activated and there is high and rapid oxygen consumption, referred to as oxidative respiratory burst response (Amulic et al., 2012; Hurst, 2012). The enzyme complex responsible for this process is called the NADPH oxidase (Dinauer, 2014). It is composed of two transmembrane proteins (p22phox and gp91phox/ NOX2, which form the cytochrome b558), three cytosolic proteins (p47phox, p67phox, p40phox) and a GTPase (Rac1 or Rac2)(Hurtado-Nedelec et al., 2014). All these components assemble at the membrane upon cell activation to form a functional electron-transfer system, which ferries electrons over the membrane from cytosolic NADPH to O<sub>2</sub>, forming superoxide (O<sub>2</sub><sup>-</sup>) (Parker et al., 2014). Superoxide is then converted to hydrogen peroxide, HOCl — in the presence of neutrophil myeloperoxidase —, or other microbicidal oxidants that synergize with granule proteins to kill internalized microorganisms (Dinauer, 2014). A common bacterial strategy to survive the oxygen-dependent antimicrobial attack from the neutrophils is the inhibition, evasion or neutralization of the NADPH oxidase (Allen, 2003; Allen et al., 2005).

If the neutrophil encounters a particulate stimulus, the NADPH oxidase complex will be activated in the phagosome membrane, releasing oxygen radicals inside this organelle (Iversen et al., 2016). Conversely, if neutrophils encounter soluble stimuli, the NADPH oxidase complex will assemble at the cell plasma membrane and the oxygen radicals will be generated and released extracellularly, which also promotes tissue damage. Furthermore,



neutrophils are also able to produce significant amounts of intracellular ROS even in the absence of phagosome formation (Parker et al., 2014).

Since 80–85 % of the flavocytochrome  $b_{558}$ , a component of the NADPH oxidase, is localized in the membrane of neutrophil specific granules the intracellular (non-phagosomal) ROS could be explained by the NADPH-oxidase activation in the granule membranes (Parker et al., 2014).

Another neutrophil killing mechanism, which does not involve oxygen consumption, relates to the maturation of the phagosome achieved by the recruitment of neutrophil granules containing microbicidal peptides (Quinn, 2014). These granules fuse with the phagosome or the plasma membrane, releasing their components to aid in the elimination of microorganisms either inside the bacteria-containing phagosome or to the extracellular environment respectively (Johnson & Criss, 2013b). Additionally, granule exocytosis contributes to many neutrophil functions, including adhesion, enhanced migration, chemotaxis, and phagocytosis (Herrero-Turrión et al., 2008; Uriarte et al., 2008).

Neutrophils have four granule subtypes, differentiated based on their density and granule content: azurophilic, specific, and gelatinase granules, as well as secretory vesicles. The azurophil granules, also known as primary or peroxidase-positive granules, are the first formed during neutrophil maturation and the largest of the granule subtypes (approx.  $0.3 \mu\text{M}$ ) (Amulic et al., 2012). These granules contain myeloperoxidase (MPO), which is

fundamental for the production of hypochlorous acid, the most potent antimicrobial compound produced in neutrophil phagosomes (Segal et al., 2000). In addition, their cargo includes  $\alpha$ -defensins, lysozyme, bactericidal/permeability increasing protein (BPI), and a number of serine proteases like neutrophil elastase (NE), proteinase 3 (PR3), neutrophil serine protease 4 (NSP4) and cathepsin G (Amulic et al., 2012; Borregaard & Cowland, 1997; Perera et al., 2013; Uriarte et al., 2016). Therefore, they are considered the most potent of the granule subtypes and function as main supply of the neutrophil's antimicrobial cargo (Amulic et al., 2012; Nordenfelt & Tapper, 2011).

Another granule subtype is the secondary or specific granules, which are characterized by their content of antimicrobial peptides such as lactoferrin and lysozyme. In addition, they provide 60 % of the membrane components of the multi-complex enzyme responsible for the respiratory burst response (Scott & Krauss, 2012). They are known to promote the neutrophil's phagocytic potential by providing CR3 (CD11b/CD18) (Borregaard & Cowland, 1997). The specific granules contain several proteins required for adhesion and extravasation of human neutrophils (Herrero-Turrión et al., 2008; Rørvig et al., 2013; Scott & Krauss, 2012).

The gelatinase or tertiary granules contain fewer antimicrobials compared to the other granule subtypes, yet they serve as a reservoir for metalloproteases (such as gelatinase and leukolysin) and contain receptors involved in directed migration (Amulic et al., 2012; Nordenfelt & Tapper, 2011). The

gelatinase exocytosis is critical for neutrophil migration towards the inflammation site since it aids in basement membrane degradation by targeting type IV collagen (Borregaard & Cowland, 1997; Nordenfelt & Tapper, 2011).

In contrast to the previously mentioned granules, which bud from the Golgi apparatus, the secretory vesicles (fourth granule subtype) have a different origin; they are formed through endocytosis during neutrophil maturation (Amulic et al., 2012). The secretory vesicles are the most readily mobilized granules and their contents are important in the early phases of inflammation (Borregaard & Cowland, 1997). While their multiple membrane-associated proteins promote neutrophil attachment to the activated endothelium during neutrophil migration, their complement and fMLF receptors, are necessary for host-pathogen interactions (Amulic et al., 2012; Herrero-Turrión et al., 2008; Nordenfelt & Tapper, 2011).

In response to inflammatory signals, each of the four granules types demonstrates a different predisposition for mobilization: the azurophilic granules are the most difficult to mobilize, followed by specific granules, gelatinase granules, and secretory vesicles being the most easily mobilized (Amulic et al., 2012). The accurate regulation of granule mobilization determines in great part the timing and targeting of neutrophil responses, ensures efficient delivery of granule content and prevents tissue damage to the host (Nordenfelt & Tapper, 2011).

In situations where the intruder cannot be phagocytized and

extracellular responses are not able to eradicate it, neutrophils release their DNA content forming extracellular traps (NETs) of decondensed chromatin and antimicrobial granule proteins as the last attempt to clear infection (Amulic et al., 2012; Brinkmann et al., 2004; Brinkmann & Zychlinsky, 2012). So far, the mechanism of NET formation is not completely understood. Neutrophils can expulse their mitochondrial DNA while remaining viable, as a quick response (within 30 min) after moderate stimulation (Yousefi et al., 2009). Contrariwise, after stronger and longer stimulation, neutrophils can undergo NETosis, as a unique form of programmed cell death that involves nuclear DNA (Papayannopoulos & Zychlinsky, 2009; Uriarte et al., 2016; White et al., 2016).

In chronic inflammatory diseases such as periodontitis, pathogens have evolved mechanisms to evade neutrophil clearance (Berezow & Darveau, 2011). For example, several periodontal pathogens such as *P. gingivalis* (Berezow & Darveau, 2011), and *T. denticola* (Sela, 2001) are resistant to oxidative killing. Moreover, *Filifactor alocis* not only fails to induce ROS response in neutrophils, but it also prevents granule recruitment to the bacteria-containing phagosome (Uriarte et al. unpublished observations). Furthermore, it has been suggested that *T. forsythia* secretes DNase, an enzyme able to degrade nucleic acids, as a mechanism to escape neutrophil extracellular traps (Amulic & Hayes, 2011; Palmer et al., 2012; Uriarte et al., 2016).

## CHAPTER 2

### RESEARCH HYPOTHESIS AND SPECIFIC AIMS

Recent advancements in the periodontal research field attribute the etiology of periodontal diseases to a synergistic and dysbiotic microbial community, where different microorganisms play diverse roles to modulate the host responses and disrupt the oral homeostasis (Hajishengallis & Lamont, 2012). These interactions between the oral microbiota and the innate immune system are key determinants of oral health status. An association between the newly appreciated species *Peptoanaerobacter stomatis* and periodontitis has recently emerged (Kumar et al., 2005; Murphy & Frick, 2013; Sizova et al., 2015). However, since its possible pathogenic mechanisms have not been investigated, the role it plays in periodontal diseases remains unknown. Therefore, the gap in knowledge of how *P. stomatis* putative virulence factors interact with the key host response is considered the narrow research problem for this project.

The overall objective of this discovery science approach is to characterize the human neutrophil antimicrobial responses towards *P.stomatis*.

To this end, studying the interactions between neutrophils and *P. stomatis* will provide valuable information to understand the role of this pathogen in periodontal diseases and enhance our understanding of bacterial strategies to subvert innate immunity.

### Research Hypothesis

*P. stomatis* induces human neutrophil killing mechanisms.

### Specific Aims

Specific Aim 1: To define *P. stomatis*' susceptibility to neutrophil oxidative killing.

Hypothesis: *P. stomatis* challenge will stimulate the neutrophil respiratory burst response.

Specific Aim 2: To characterize the human neutrophil granule response to *P. stomatis* challenge.

Hypothesis: *P. stomatis* challenge will stimulate neutrophil activation.

## CHAPTER 3

### METHODS

In this experimental model study, neutrophils isolated from healthy human blood donors were unstimulated, or challenged with *Peptoanaerobacter stomatis* strain CM2 at a multiplicity of infection (MOI) of 10 (unless otherwise noted), or with *Staphylococcus aureus* at a MOI of 10, which was used as a positive control.

#### Study approval

The Institutional Review Boards at the University of Louisville approved the use of human donors for this study.

#### Human neutrophil isolation

Neutrophils were isolated from healthy human donors using plasma-Percoll gradients (Uriarte et al., 2011). Microscopic evaluations using Wright

staining (ENG Scientific, Inc) showed that >90% of isolated cells were neutrophils for each experiment. Unidentified samples from 3 different donors' neutrophils on different days were pooled for each independent experiment (unless otherwise noted).

### Bacteria Preparation

*P. stomatis* strain CM2 (provided by Dr. Slava Epstein, Northeastern University) was cultured anaerobically at 37°C in Tryptic Soy Broth medium supplemented with 20 g/L yeast extract, 1.0% hemin and 1.0% reducing agent (37.5 g/L NH<sub>4</sub>Cl, 25 g/L MgCl<sub>2</sub> x 6H<sub>2</sub>O, 5 g/L CaCl<sub>2</sub> x 2H<sub>2</sub>O, 50 g/L L-cysteine HCl, 5 g/L FeCl<sub>2</sub> x 4H<sub>2</sub>O).

Non-viable bacteria were prepared by 90% isopropanol treatment after incubation for 10 min at room temperature. In order to remove residual isopropanol, the bacteria were washed twice in PBS buffer. Bacteria was cultured in broth at 37°C for 24hrs to confirm non-viability before use.

For fluorescence assays, *P. stomatis* was labeled either with DAPI (10 µg/ml ) or with CFSE (0.25mg/ml) for 30 min at room temperature in the dark and washed 3 times with PBS prior to its use.



## Phagocytosis of *P. stomatis* by human neutrophils

Human neutrophils were stimulated with CFSE labeled human serum opsonized *P. stomatis*, non-opsonized *P. stomatis*, or with AlexaFluor488 labeled human serum opsonized *S. aureus*, or non-opsonized *S. aureus*. Cells were incubated in a shaking water bath at 37°C for 30 min or 120 min. Cells were rinsed with 0.05% sodium azide and fixed with 1% paraformaldehyde. Images were obtained, quantified and visualized using Amnis Imagestream X. Bacterial internalization (CFSE fluorescence) was detected using a 488 nm solid state laser. 1000 neutrophil events were collected per condition and sorted into bacteria positive or negative bins based on CFSE / AlexaFluor488 intensity and a mask designed to ignore signals from associated or extracellular bacteria. Data are expressed as the mean  $\pm$  SEM of the percent of bacteria positive neutrophils.

As an approach to differentiate more precisely the bacteria that is internalized from the one that is associated to the neutrophil, we used Z-stacks to analyze the cell as a 3D image by confocal microscopy. Human neutrophils were stimulated with CFSE labeled *P. stomatis*, or with AlexaFluor488 labeled *S. aureus* and incubated in a shaking water bath at 37°C for 30 min. After incubation, the samples were rinsed once with RPMI media, transferred to plates containing plasma-coated coverslips, and centrifuged at 600 x g for 8 min at 4°C. Cells were fixed with 10% formalin, and DAPI was used as a nuclear stain. Images were acquired by confocal microscopy. To quantify the percent of infection, 200 neutrophils were tallied per condition and were classified as

bacteria positive or bacteria negative cells based on CFSE fluorescence.

#### Bacterial viability assessed by colony-forming unit (CFU) assays

Human neutrophils ( $4 \times 10^6$  cells/ml) were unstimulated or stimulated with *P. stomatis* at 37°C for 30 min. After incubation, the infected neutrophils were washed once with PBS and lysed with distilled H<sub>2</sub>O brought to pH 11 for 10 min to liberate the bacteria. The lysates and supernatants were collected in different tubes before proceeding to dilute and plate each condition on TY agar medium. Samples were incubated under anaerobic conditions and colonies were counted after 6 days of culture. Bacteria not exposed to neutrophils were used as a growth control.

#### Bacterial viability assessed by BacLight with synchronized phagocytosis

Human neutrophils were settled on human serum coated coverslips, incubated in RPMI media and stimulated with DAPI-labeled viable *P. stomatis* or with DAPI labeled non-viable *P. stomatis* (used as a control). Viable and non-viable *P. stomatis*, in the absence of host cells, were also used as staining controls. Samples were centrifuged at 600 x g, 14°C for 4 min in order to force neutrophil bacterial internalization, and incubated at 37°C with 5% CO<sub>2</sub> for 30 min or 120 min. After incubation, the membrane impermeable dye SYTOX

Green (0.4 mM) in 0.1 MOPS pH 7.2, 1 mM MgCl<sub>2</sub> (MOPS/MgCl<sub>2</sub>) was added to detect bacterial cells with a compromised cell membrane.

Confocal mages were acquired within 30 min using a Fluoview FV1000 confocal microscope (Olympus) controlled by FV-10ASW software (Olympus). SYTOX Green (non-viable bacteria) was detected using a Multi-line Argon Laser (488 nm), 30mW with an emission of 515 - 555 nm, and DAPI (viable bacteria) was detected using a diode violet laser (405 nm), 25 mW with an emission of 400 nm. Within neutrophils, both live and a mix of viable and non-viable bacterial cells were observed based on their staining. Quantification was performed by totaling the viable (blue) and non-viable (green) intracellular and extracellular bacteria of 100 neutrophils in 3 independent experiments (Johnson & Criss, 2013a).

#### Bacterial viability assessed by BacLight in suspension

Human neutrophils ( $2 \times 10^6$  cells/ml) were exposed with DAPI-labeled viable *P. stomatis* or with DAPI labeled non-viable *P. stomatis* (as a control) in a shaking water bath at 37°C for 30 min or 120 min. At each time point, the samples were transferred to plates containing plasma-coated coverslips and centrifuged at 600 x g for 8 min at 4°C. The membrane-impermeable dye SYTOX Green (0.4 μM) in 0.1 MOPS pH 7.2, 1 mM MgCl<sub>2</sub> was added to detect bacterial cells with a compromised cell membrane. Confocal images were

acquired, analyzed and quantified as previously described (Johnson & Criss, 2013a).

#### Effect of *P. stomatis* on intracellular respiratory burst response

The ability of challenged neutrophils to mount an appropriate respiratory burst response was tested by measuring neutrophil intracellular reactive oxygen species production. Neutrophils were challenged with *P. stomatis*, or with *S. aureus* (used as a positive control). To measure H<sub>2</sub>O<sub>2</sub> production, neutrophils were treated with 5 μM 2',7'-dichlorofluorescein (DCF) for 10 min, followed by bacterial challenge with the respective pathogen (MOI 1, 2.5, 5 or 10) for 15 min or 30 min. The phagocytosis-stimulated respiratory burst was measured by DCF oxidation, analyzing the samples by flow cytometry using a BD FACSCalibur™. Data were expressed as the mean channel of fluorescence (mcf).

#### NBT Oxidation at the phagosome

In order to determine the amount and specific location of intracellular superoxide anion (O<sub>2</sub><sup>-</sup>) produced by human neutrophils after challenge with *P. stomatis*, a colorimetric assay using nitroblue tetrazolium (NBT) was utilized. Neutrophils (1 x 10<sup>6</sup> cell/ml) attached to plasma-coated glass coverslips were unstimulated or stimulated with *S. aureus* or with *P. stomatis*. Phagocytosis was

synchronized at 600 x g 14°C for 4 min and cells were incubated at 37°C with 5% CO<sub>2</sub> for 60 min in RPMI containing NBT. After incubation, cells were fixed with methanol and analyzed by light microscopy. Reduced NBT precipitates were visualized as blue granules of formazan.

#### Effect of *P. stomatis* on extracellular respiratory burst response

Extracellular production of reactive oxygen species (ROS) was measured by the colorimetric reduction of ferricytochrome C. Human neutrophils (4x10<sup>6</sup> cells/ml) were left unstimulated or were challenged with fMLF (300 nM) for 5 min, or with *P. stomatis* for 5, 15, or 30 min at 37°C. For neutrophil priming assays, neutrophils were pretreated with TNF- $\alpha$  (2ng /ml) for 10 min, or with *P. stomatis* for 10 min, followed by stimulation with fMLF (300 nM) for 5 min. After stimulation, the samples were centrifuged for 10 min at 600 x g, 4 °C and supernatants were collected. The superoxide anion release was measured spectrophotometrically at 550 nm as the superoxide dismutase-inhibitable reduction of ferricytochrome C. Data were expressed as nmol/mL of superoxide release using the formula  $x-bkgd/0.021 = [O_2]^- \text{ nM}$ .

## Immunostaining and confocal microscopy for specific and azurophil granule recruitment

The fusion of specific and azurophil granules with bacteria-containing phagosomes was tested by immunostaining using confocal microscopy as previously described is (Allen et al., 2005; Johnson & Criss, 2013b; Monfregola et al., 2012). Human neutrophils were settled on human serum coated coverslips and incubated in RPMI 1640 with 10% fetal bovine serum and stimulated with bacteria for 30 min. The analyzed conditions for this experiment were neutrophils challenged with CFSE- labeled *P. stomatis*, or *S. aureus* AF488 (used as positive control), or unchallenged neutrophils (used as a negative control). Neutrophils were fixed, permeabilized and labeled with a specific granule marker (anti-lactoferrin primary antibody), or an azurophil granule marker (anti-elastase antibody). ALEXA Fluor 555 anti-rabbit secondary antibody was used for visualization of both granule markers, and DAPI was used as a nuclear stain.

Confocal images were obtained using a Fluoview FV1000 confocal microscope (Olympus) controlled by FV-10ASW software (Olympus). Alexa Fluor 555 (granule markers) imaging was detected using a HeNe Laser (543 nm), 1 mW with an emission of 600 - 660 nm. CFSE (bacteria) was detected using a Multi-line Argon Laser (488 nm), 30 mW with an emission of 515 - 555 nm. DAPI (nucleus) was detected using a diode violet laser (405 nm), 25 mW with an emission of 400 nm. Z-stacks (10  $\mu$ m) of the challenged neutrophils were

used to determine specific and azurophil granule fusion to bacteria-containing phagosomes. To quantify the percent of granule fusion to pathogen-containing phagosomes, 100 cells were counted per condition and if  $\geq 50\%$  of the phagosome was surrounded by lactoferrin or elastase, it was considered positive for that granule marker (Allen et al., 2005; Johnson & Criss, 2013b).

### Neutrophil granule exocytosis

The exocytosis of azurophil granules, specific granules, and secretory vesicles was determined by measuring the increase in plasma membrane expression of membrane-associated receptors CD63, CD66b, or CD35 respectively. Isolated human neutrophils ( $4 \times 10^6$  cells/ml) were suspended in Krebs+ buffer and were incubated at 37°C. The experimental conditions for this experiments were neutrophils challenged with *P. stomatis* or *S. aureus* (positive control) at different multiplicities of infection (10, 50, or 100) for 30 min; or with fMLF (300 nM) for 5 min (positive control); or pretreated with TNF- $\alpha$  (2 ng/mL) for 10 min followed by a fMLF stimulus for 5 min (positive control); or unstimulated neutrophils (negative control). After the bacterial challenge, cells were incubated on ice for 45 min with the following antibodies: (FITC)-conjugated anti-CD63 (azurophil granule marker), or PE-conjugated anti-human CD35 (secretory vesicle marker), or FITC-conjugated anti-CD66b (specific granule marker). The samples were washed with 0.5% sodium azide, fixed with 1% paraformaldehyde, and analyzed by flow cytometry using a BD FACSCalibur™. Data was

expressed as the mean channel of fluorescence (mcf).

A commercially available enzyme-linked immunosorbent assay (R&D systems, Minneapolis, MN, USA) was used to determine the neutrophil exocytosis of gelatinase granules by measuring the amount of matrix metalloprotease (MMP)-9 present in the supernatants of the stimulated neutrophils. Human neutrophils ( $4 \times 10^6$  cells/ml) were either left unstimulated or challenged with *P. stomatis* for 15, 30 or 60 min; or with fMLF (300 nM) for 5 min. Samples were centrifuged at 6000 x g for 30 sec, and supernatants were collected, mixed with a 1% protease inhibitor, and stored at -80 °C until use. The samples were analyzed by ELISA and data were expressed as mean  $\pm$  SEM of nanograms of MMP-9 released in the collected supernatants.

### Statistical Analysis

The differences among experimental conditions and time points were analyzed by one-way analysis of variance (ANOVA) and the post-test Tukey multiple-comparison or by two-tailed Student's t-test using GraphPad Prism Software. Differences were considered significant at the level  $P < 0.05$ .



## CHAPTER 4

### RESULTS

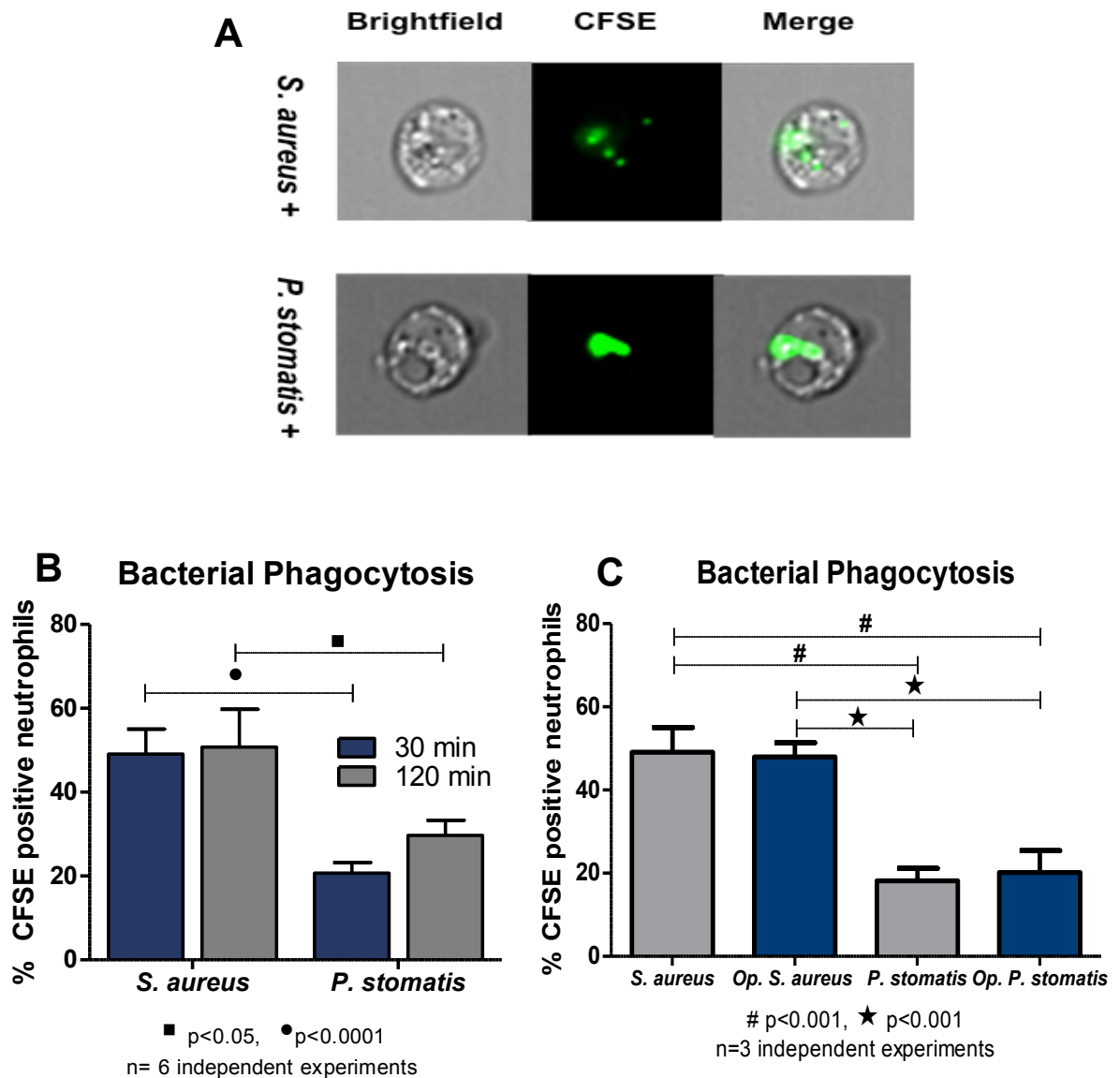
Specific Aim 1: To define *P. stomatis*' susceptibility to neutrophil oxidative killing.

Hypothesis: *P. stomatis* challenge will stimulate the neutrophil respiratory burst response.

Neutrophils are professional phagocytes in charge of maintaining the gingival crevice homeostasis and protecting the periodontal tissues from infection. In order to perform this protective function, neutrophils possess different killing mechanisms to eliminate intruders. The respiratory burst response with the generation of ROS and the release of the microbicidal molecules contained in the neutrophil granules are considered major defense mechanisms for neutrophil bacterial clearance in the gingival crevice. Consequently, the pathogenic mechanisms of many microorganisms include immune evasion. Therefore, we evaluated the phagocytosis of *P. stomatis* and the subsequent production of ROS.

### Neutrophils had a low phagocytic efficiency for *P. stomatis*

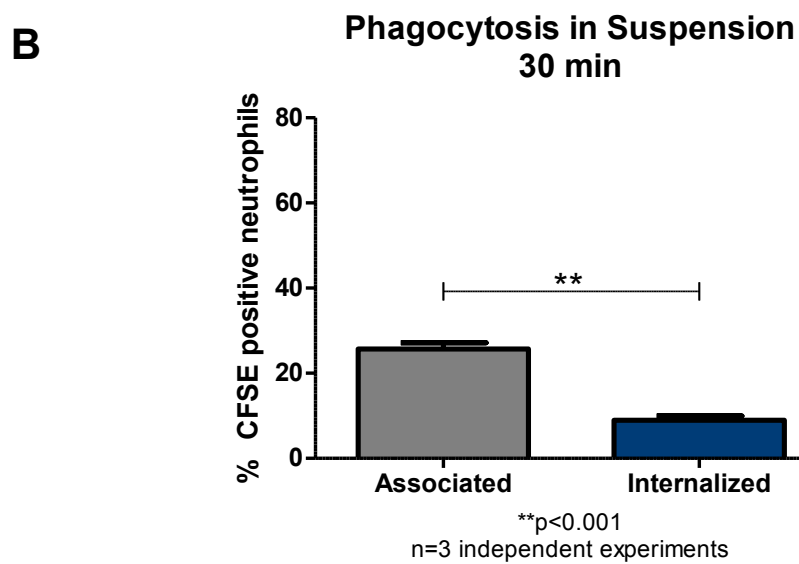
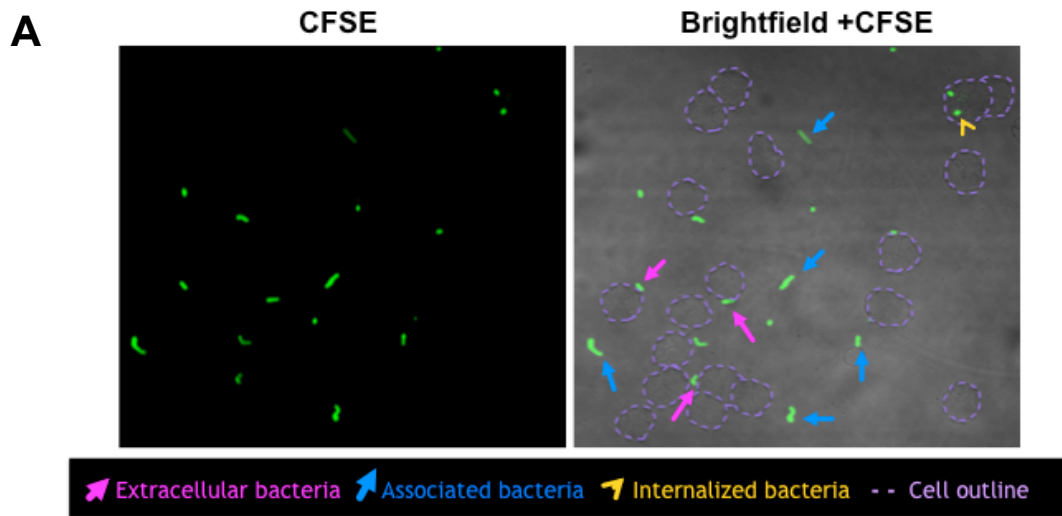
To determine the susceptibility of *P. stomatis* to neutrophil killing, we analyzed the percentage of cells in suspension that engulfed bacteria was analyzed by imaging flow cytometry (Fig. 1A). After a 30 min challenge, only 21% of the neutrophils had internalized *P. stomatis*, which suggests that the oral bacteria were more defiant to phagocytosis than the positive control *S. aureus* (49% internalized), (Fig. 1B). Neutrophils can recognize both non-opsonized as well as opsonized bacteria, with an enhanced phagocytic ability when the microbe is opsonized. Hence we wanted to determine if serum opsonization would increase neutrophil phagocytic ability towards *P. stomatis*. However, the phagocytic efficiency of neutrophils challenged with opsonized *P. stomatis* after 30 min (20%) was similar to the percent observed when the cells were challenged with non-opsonized bacteria (Fig. 1C). Moreover, there was no significant increase in phagocytosis over time; after a 120 min challenge, only 30% of neutrophils had internalized *P. stomatis* (Fig. 1B).



**Figure 1A-1C.** *P. stomatis* phagocytosis by human neutrophils analyzed by imaging flow cytometry.

Internalization of CFSE labeled opsonized *P. stomatis* (Op Ps), non-opsonized *P. stomatis* (Ps) opsonized *S. aureus* (Op Sa), or non-opsonized *S. aureus* (Sa) was visualized (A) and quantified (B and C) at 30 min and 120 min via ImageStreamX imaging flow cytometry. 1000 neutrophil events were collected and sorted into bacteria positive or negative bins based on CFSE / AlexaFluor488 intensity and a mask designed to ignore signals from associated or extracellular bacteria. Data are expressed as the mean  $\pm$  SEM of the percent of bacteria positive neutrophils from 6 independent experiments. (B) ● p< 0.0001 compared to *S. aureus* 30 min, ■ p< 0.05 compared to *S. aureus* 120 min. (C) # p< 0.001 compared to *S. aureus* 30 min, ★ p< 0.001 compared to Opsonized *S. aureus* 30 min. .

The data obtained by imaging flow cytometry suggest that *P. stomatis* resist neutrophil phagocytosis. To further confirm these results, bacteria uptake by suspension neutrophils challenged with CFSE-labeled *P. stomatis* was analyzed by confocal microscopy. Images were visualized in 10  $\mu\text{m}$  Z-stacks and the cells were categorized as neutrophils that had internalized, associated or no bacteria based on bacteria CFSE fluorescence and the neutrophil brightfield. The confocal microscopy data mirrored the flow cytometry results with values of 9% bacteria internalization (after 30 min), and values of 36% including both infected neutrophils and cells with surface-bound bacteria (Fig. 2A – B). These results suggest that *P. stomatis* is able to avoid phagocytosis.

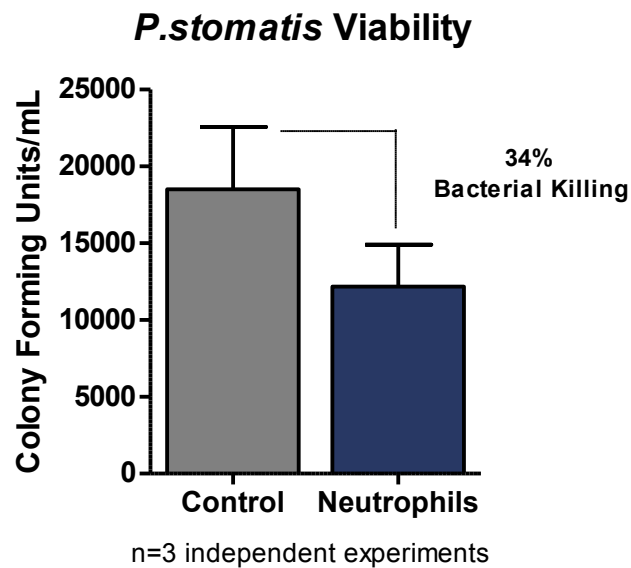


**Figure 2A – 2B.** *P. stomatis* phagocytosis by human neutrophils analyzed by confocal microscopy

Internalization of CFSE labeled *P. stomatis* was visualized by confocal microscopy (A) and quantified (B) after 30 min. 200 cells were counted and sorted into neutrophils with internalized bacteria, neutrophils with associated bacteria, and uninfected neutrophils. Data are expressed as the mean ± SEM of the percent of bacteria positive neutrophils from 3 independent experiments. \*\*p<0.001 compared associated vs internalized bacteria.

## Bacterial viability

To establish the survival rate of *P. stomatis* after exposure to human neutrophils, colony forming units (CFU) assays were performed. *P. stomatis* showed an overall survival of 66% after being exposed to neutrophils for 30 min. These results suggest that after 30 min the neutrophils efficiency to eliminate *P. stomatis* is limited (Fig. 3).

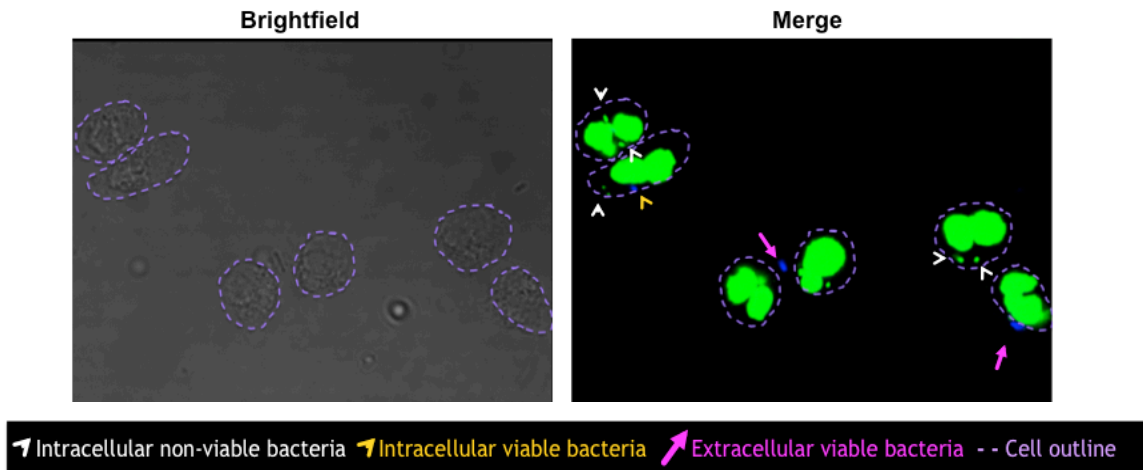


**Figure 3. *P. stomatis* viability after 30 min neutrophil exposure**

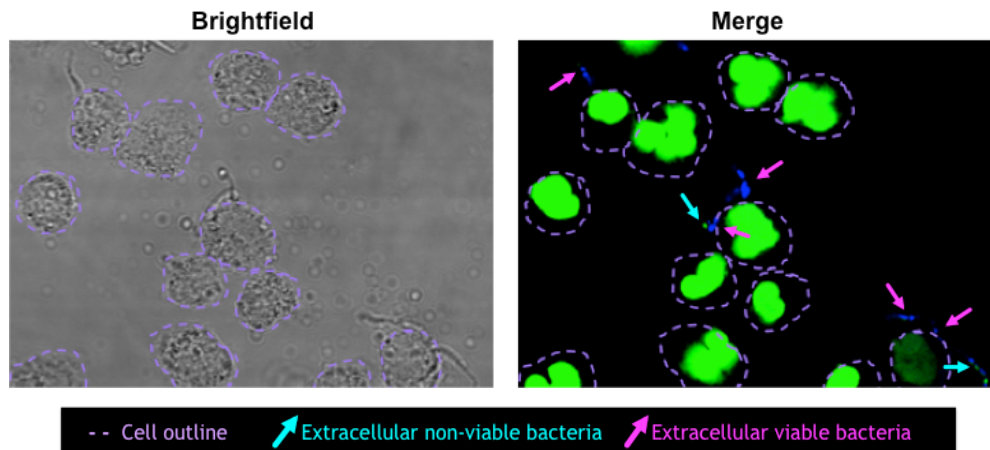
Human neutrophils were challenged with *P. stomatis* (neutrophils) and incubated at 37°C for 30 minutes. After incubation, the infected neutrophils were lysed with distilled water brought to pH 11, diluted and plated on TY agar medium. Samples were incubated under anaerobic conditions and colonies were counted after 6 days of culture. Bacteria not exposed to neutrophils were used as growth control (control). Data are expressed as the mean ± SEM of colony forming units per from 3 independent experiments. There was no significant difference between the analyzed conditions.

To determine the bacterial killing of attached to, internalized or intracellular bacteria by host cells and to assess if the low phagocytic ability of neutrophils towards *P. stomatis* seen in the previous assays was mirrored by bactericidal activity; BacLight assays with and without synchronized phagocytosis were performed on neutrophils challenged with DAPI-labeled *P. stomatis*. In this protocol viable bacteria appeared blue and nonviable appeared green or turquoise (blue + green). It is important to note that SYTOX Green is a DNA dye which stains both the bacteria and the neutrophil nucleus (Fig. 4A, 4B).

**A** BacLight after *P. stomatis* challenge with synchronized phagocytosis



**B** BacLight after *P. stomatis* challenge without synchronized phagocytosis

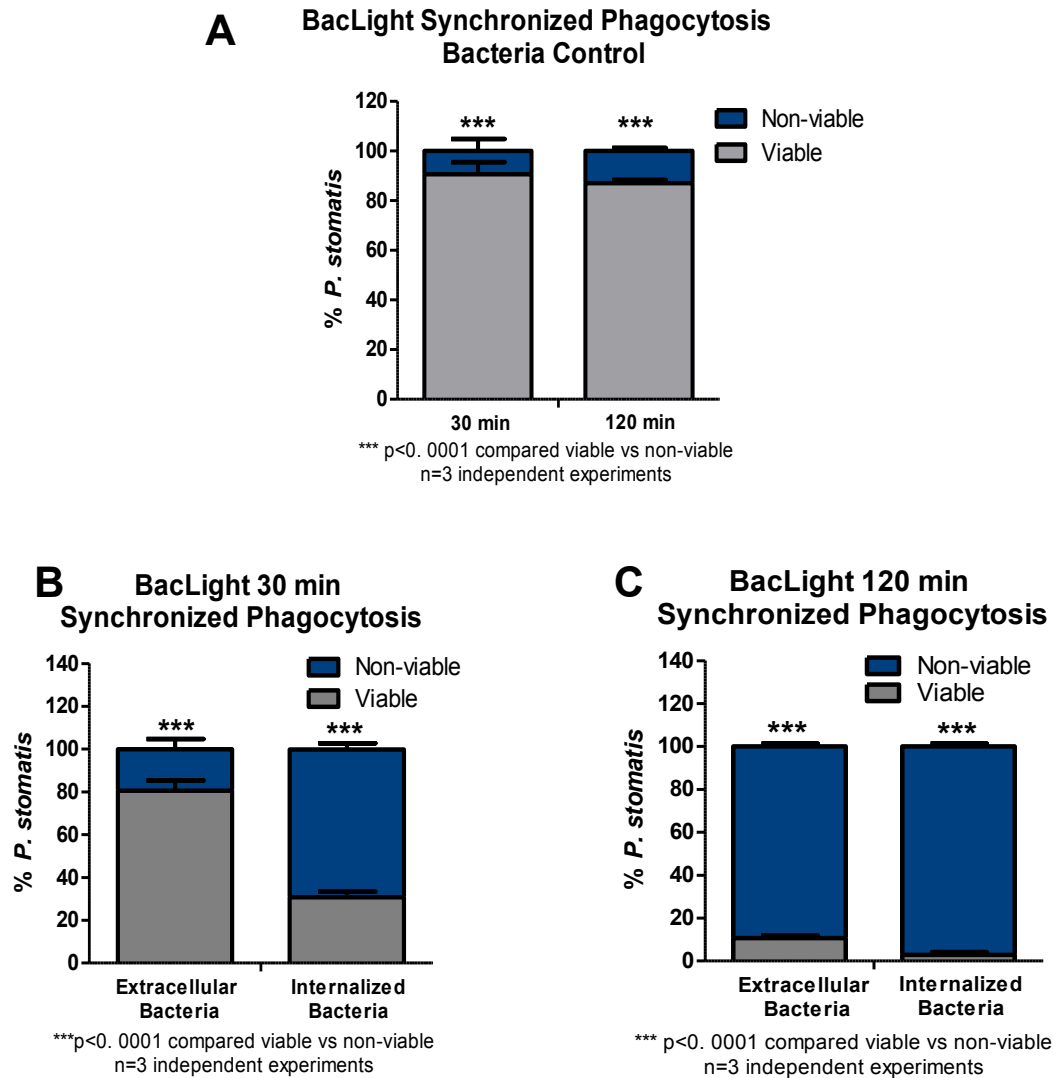


**Figure 4A -4B. BacLight confocal microscopy images**

Neutrophils were infected with *P. stomatis* with synchronized phagocytosis (A) or without it (B) and incubated for 30 min. Viable *P. stomatis* (blue) and non-viable *P. stomatis* (green) were discriminated using BacLight viability dyes SYTOX Green and DAPI. In (A) and (B) arrow heads indicate viable bacteria, and arrows indicate non-viable bacteria. Images were obtained by confocal microscopy and 200 neutrophils were analyzed.



After synchronized phagocytosis and 30 min of bacterial challenge, 80% of the attached neutrophils were able to engulf *P. stomatis*. From this percentage of internalized bacteria the majority was killed (69%), while 81% of the bacteria that was not engulfed remained viable (Fig. 5B). After 120 min the percentage of viable bacteria decreased to a minimum, 11% for extracellular and 3% for internalized *P. stomatis* (Fig 5C). To account for the bacteria that could be killed or damaged due to manipulation during this assay, bacteria not exposed to neutrophils were also analyzed (Fig 5A). Together these results suggest that the radical bacteria reduction seen after the 120 min time point is actually caused by neutrophil extracellular killing. This phenomenon could probably be attributed to the neutrophil granule contents, such as matrix metalloproteinases or lysozyme, released during neutrophil degranulation.

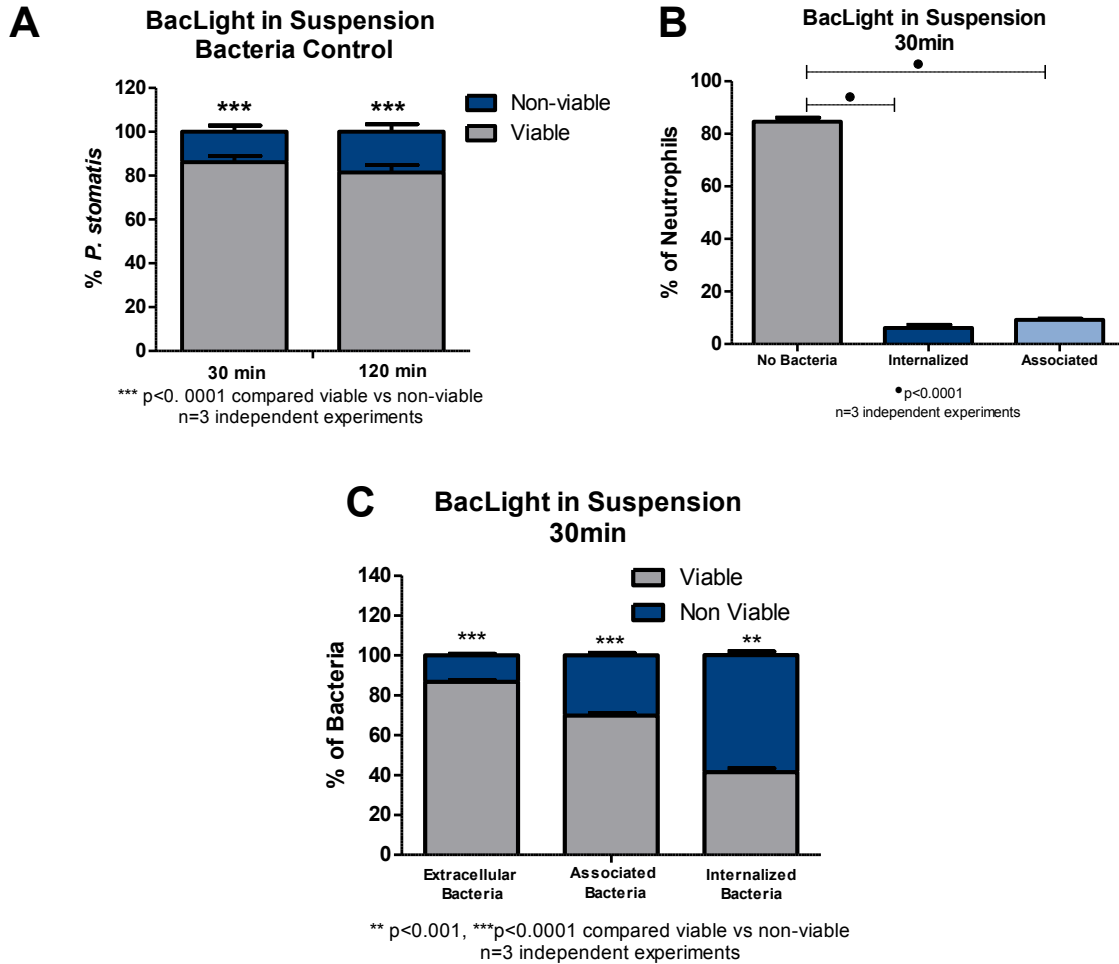


**Figure 5A -5C. *P. stomatis* viability after neutrophil challenge with synchronized phagocytosis**

Neutrophils were infected with *P. stomatis* with synchronized phagocytosis and incubated for 30 min (B) and 120 min (C). Bacteria alone was used a control (A). After incubation, viable (gray) and non-viable (blue) *P. stomatis* were discriminated using BacLight viability dyes DAPI and SYTOX Green respectively. Images were obtained by confocal microscopy and 200 neutrophils were analyzed and bacteria among those fields were counted. Data are expressed as the mean  $\pm$  SEM of the percent of intracellular, extracellular, viable and non-viable bacteria from 3 independent experiments. \*\*\*p<0.0001 compared viable vs non-viable.

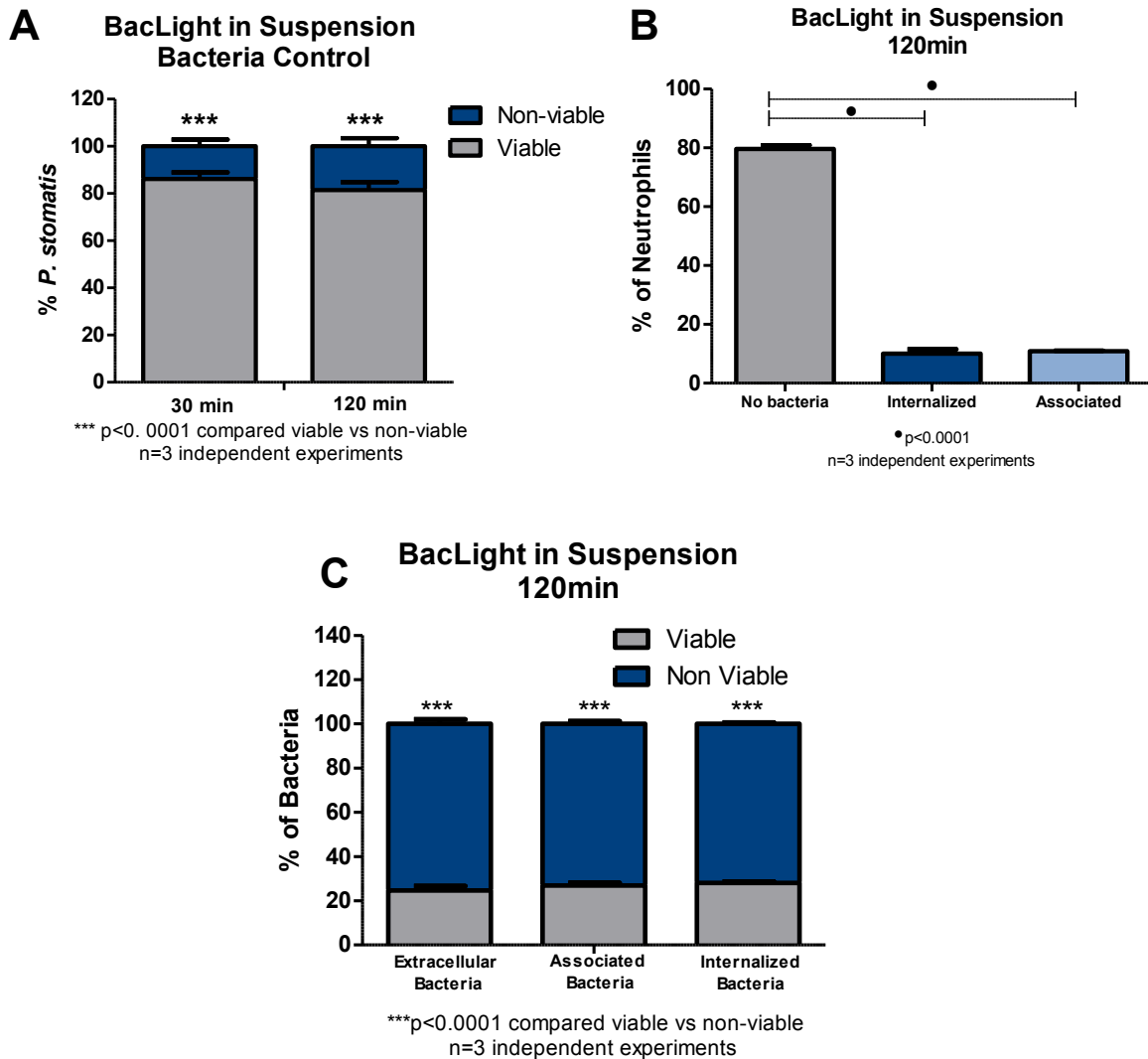
In contrast, neutrophils in suspension were hardly able to internalize *P. stomatis* after 30 min (6%), and only 59% of these intracellular bacteria was eradicated (Fig. 6A C). About 9% of the neutrophils showed bacteria attached to their membrane, from where 70% was viable (Fig. 6A C). Moreover, 87% of the extracellular *P. stomatis* remained viable (Fig. 6B C). *P. stomatis* internalization (10%) did not increase significantly after a 120 min challenge (Fig. 7B). Nevertheless, bacterial survival decreased significantly with values of only 28% internalized, 27% associated, and 25% r extracellular viable *P. stomatis* (Fig. 7B C).

These data suggest that when phagocytosis is forced and *P. stomatis* is internalized, neutrophils are able to degrade this periodontal pathogen efficiently. However if neutrophils are challenged with *P. stomatis* in suspension, a more representative scenario of the gingival sulcus, these bacteria are able to avoid phagocytosis and delay its killing.



**Figure 6A -6C. *P. stomatis* viability after a 30 min neutrophil challenge in suspension**

Neutrophils were infected with *P. stomatis* without synchronized phagocytosis and incubated for 30 min. Bacteria alone was used a control (A). After incubation, viable (gray) and non-viable (blue) *P. stomatis* were discriminated using BacLight viability dyes DAPI and SYTOX Green respectively. Images were obtained by confocal microscopy and 200 neutrophils were counted and classified as cells with internalized bacteria, cells with associated bacteria, or non-infected cells. *P. stomatis* bacteria among those fields were also quantified and classified as viable or non-viable. (B) Graph shows data expressed as the mean  $\pm$  SEM of the percent of non-infected cells, cells with internalized *P. stomatis* and cells with associated *P. stomatis* from 3 independent experiments. (C) Graph shows data expressed as the mean  $\pm$  SEM of the percent of intracellular, extracellular, viable and non-viable bacteria from 3 independent experiments. \*\*\*p<0.0001 compared viable vs non-viable.

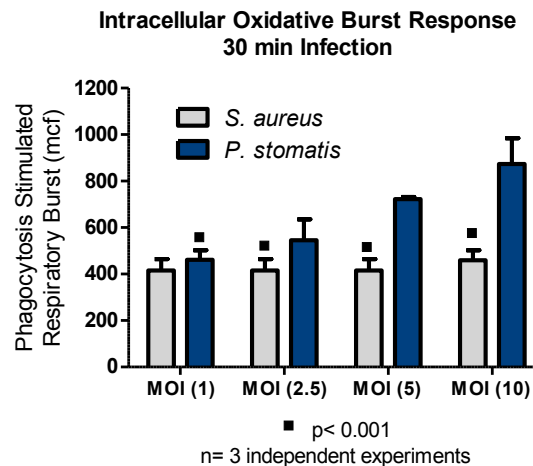


**Figure 7A-7C. *P. stomatis* viability after a 120 min neutrophil challenge in suspension**

Neutrophils were infected with *P. stomatis* without synchronized phagocytosis and incubated for 120 min. Bacteria alone was used a control (A). After incubation, viable (gray) and non-viable (blue) *P. stomatis* were discriminated using BacLight viability dyes DAPI and SYTOX Green respectively. Images were obtained by confocal microscopy and 200 neutrophils were counted and classified as cells with internalized bacteria, cells with associated bacteria, or non-infected cells. *P. stomatis* bacteria among those fields were also quantified and classified as viable or non-viable. (B) Graph shows data expressed as the mean  $\pm$  SEM of the percent of non-infected cells, cells with internalized *P. stomatis* and cells with associated *P. stomatis* from 3 independent experiments. (C) Graph shows data expressed as the mean  $\pm$  SEM of the percent of intracellular, extracellular, viable and non-viable bacteria from 3 independent experiments. \*\*\*p<0.0001 compared viable vs non-viable.

## *P. stomatis* induces a robust neutrophil respiratory burst response

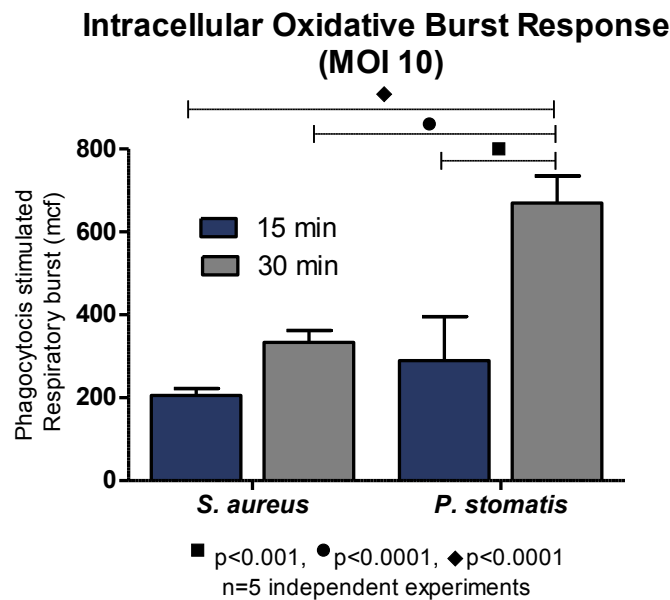
The high generation of reactive oxygen species (ROS) in neutrophils is considered a potent and effective antimicrobial weapon. However, if this response is not regulated properly it might also lead to the severe tissue destruction of the periodontium characteristic of periodontal disease. To determine the effect of *P. stomatis* on the intracellular respiratory burst response in human neutrophils, the phagocytosis-stimulated respiratory burst was measured by flow cytometry. To establish if the respiratory burst response to *P. stomatis* challenge is dependent on the bacteria to neutrophil ratio, neutrophils were challenged with this periodontal pathogen or with *S. aureus*, as a positive control, at different multiplicities of infection (1, 2.5, 5, or 10) for 30 min (Fig. 9). Both, *S. aureus* and *P. stomatis*, induced a significant respiratory burst response even at low multiplicity of infection ratios (Fig. 8).



**Figure 8. Neutrophil ROS response to *P. Stomatitis* challenge**

Neutrophils were challenged with *S. aureus* or *P. stomatis* at MOI of 1, 2.5, 5, or 10 for 30 min. The intracellular respiratory burst response was analyzed by measuring the change of fluorescence after oxidation of dichlorofluorescein diacetate (DCF) by flow cytometry. Data are expressed as mean  $\pm$  SEM of the mean channel of fluorescence (mcf) from 3 independent experiments.  $\square$  p < 0.001 compared to *P. stomatis* MOI 10.

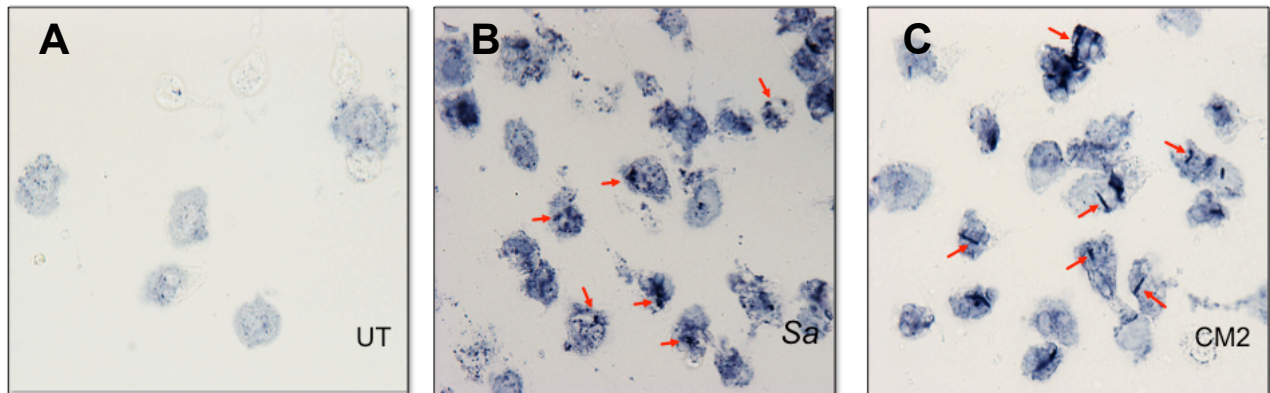
Since the greater respiratory burst response was seen at an MOI OF 10, neutrophils were challenged for 15 and 30 min to determine if this response was time dependent. Data expressed as mean  $\pm$  SEM of the mean channel of fluorescence (mcf) show that incubation with *P. stomatis* for 30 min induced a significant increase in the oxidative burst response compared to the *S. aureus* positive control, (Fig. 9).



**Figure 9. *P. stomatis* induces a robust ROS response in human neutrophils**

Neutrophils were challenged with *S. aureus* (S.a. ;MOI of 10) or *P. stomatis* (P.s. ;MOI 10) for 15 (blue) or 30 min (gray) and the intracellular respiratory burst response was analyzed by measuring the change of fluorescence after oxidation of dichlorofluorescein diacetate (DCF) by flow cytometry. Data are expressed as mean  $\pm$  SEM of the mean channel of fluorescence (mcf) from 5 independent experiments. •p< 0.0001 compared to *S. aureus* 30 min, ■p< 0.05 compared to *P. stomatis* 15 min, ◆p< 0.0001 compared to *S. aureus* 15 min.

To visualize the specific location of the intracellular ROS produced by human neutrophils after challenge with *P. stomatis*, nitroblue tetrazolium (NBT) assay was utilized. As shown in Fig. 10, more than 50% of *P. stomatis* phagosomes contained dark blue deposits, which is an indication of a robust local respiratory burst response.



**Figure 10. Nitroblue tetrazolium sedimentation after *P. stomatis* induced ROS response in human neutrophils.**

Neutrophils ( $1 \times 10^6$  cell/ml) were either unstimulated (A), stimulated with *S. aureus* (B), or *P. stomatis* (C) for 30 min in NBT media. Images were analyzed by light microscopy. Reduced NBT precipitates were visualized as blue granules of formazan where the superoxide anion ( $O_2^-$ ) was produced.

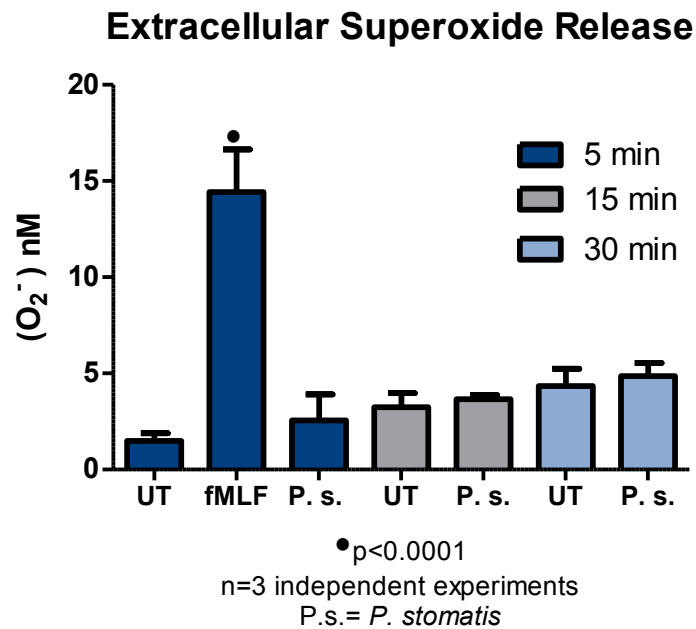
These results reveal that *P. stomatis* is able to induce a strong and localized respiratory burst response in neutrophils, even at low MOIs.



## *P. stomatis* fails to induce neutrophil superoxide extracellular release

Soluble stimuli and some pathogenic bacteria, such as *Helicobacter pylori* (Allen et al., 2005), will trigger the assembly of the NADPH oxidase at the neutrophil plasma membrane, promoting the release of ROS into the extracellular space. Increasing the superoxide concentration in the extracellular environment contributes to damage for both the host tissue and bacteria.

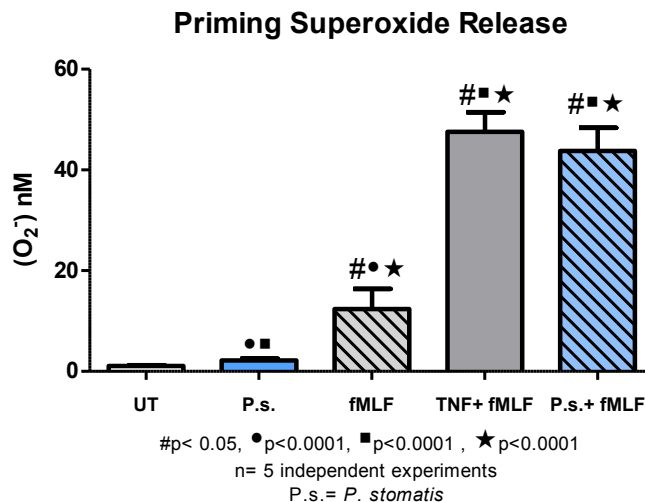
*P. stomatis* stimulation caused minimal induction of superoxide release by neutrophils (4.86 nM) independently of the time points analyzed (Fig. 11).



**Figure 11. *P. stomatis* fails to induce extracellular superoxide release**

Extracellular production of reactive oxygen species (ROS) was measured by the colorimetric reduction of ferricytochrome C in the supernatants of either unstimulated (UT) neutrophils, or stimulated with fMLF (fMLF), or with *P. stomatis* (Ps) for 5, 15 and 30 min. Data are expressed as the mean  $\pm$ SEM of nmol/mL of superoxide release from 3 independent experiments. •  $p < 0.0001$  compared to all the analyzed conditions.

Priming of neutrophils is understood as the enhanced functional response of the cell to a secondary stimulus as a result of a previous exposure with an agent that by itself is a poor agonist (Condliffe et al., 1998). To determine if *P. stomatis* would be able to prime neutrophils for an enhanced respiratory burst response, neutrophils were challenged with *P. stomatis*, followed by fMLF stimulation. The TNF $\alpha$  concentration used in this assay (200 ng/ml) is considered a priming concentration shown to not activate neutrophils, but to induce minimum extracellular ROS response, as previously described (McLeish et al., 1998; Ward et al., 2000). As shown in Fig 12, *P. stomatis* induced a significant increase of fMLF-stimulated superoxide release (47.77 nM) compared to fMLF alone (12.35 nM). Moreover, *P. stomatis* was able to prime neutrophils to the same degree as the well-documented priming agent, TNF $\alpha$  (Fig 12).



**Figure 12. *P. stomatis* neutrophil priming**

The priming of neutrophils and their subsequent extracellular production of reactive oxygen species (ROS) was measured by the colorimetric reduction of ferricytochrome C in the supernatants of neutrophils pretreated with TNF- $\alpha$  for 10 min (TNF+fMLF), or with *P. stomatis* for 10 min (P.s.+fMLF), followed by stimulation with fMLF for 5 min. Neutrophils stimulated with *P. stomatis* (P.s) or left unstimulated (UT) were used as negative controls. Data are expressed as the mean  $\pm$ SEM of nmol/mL of superoxide release for 5 independent experiments. #p< 0.05 compared to UT, •p< 0.0001 compared to TNF+fMLF, ■p< 0.0001 compared to fMLF, , ★ p< 0.001 compared to *P. stomatis*.

These results reveal that *P. stomatis* is a poor inducer of extracellular ROS response: however it can modulate the respiratory burst response to other stimuli.

### Neutrophil granule response

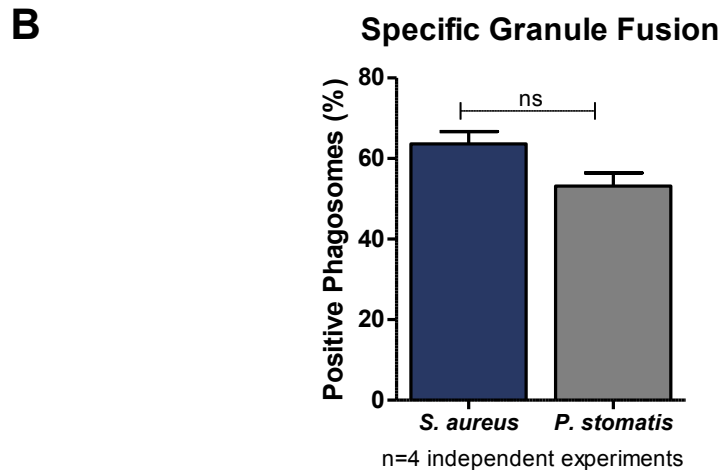
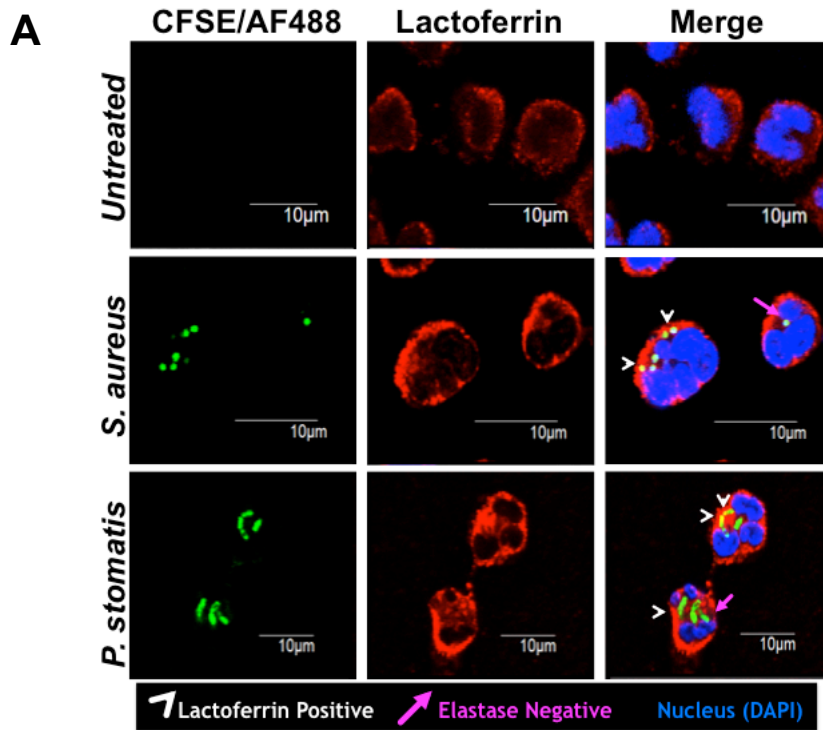
Besides the respiratory burst response, neutrophils have another potent antimicrobial strategy to withstand intruders. Neutrophils contain different granule subtypes involved in both intracellular and extracellular bacterial killing. As the pathogen-containing phagosome matures, granules containing microbicidal peptides fuse releasing their cargo into the phagosome and/or undergo exocytosis.

Specific Aim 2: To characterize human neutrophil granule response to *P. stomatis* challenge.

Hypothesis: *P. stomatis* challenge will stimulate neutrophil activation.

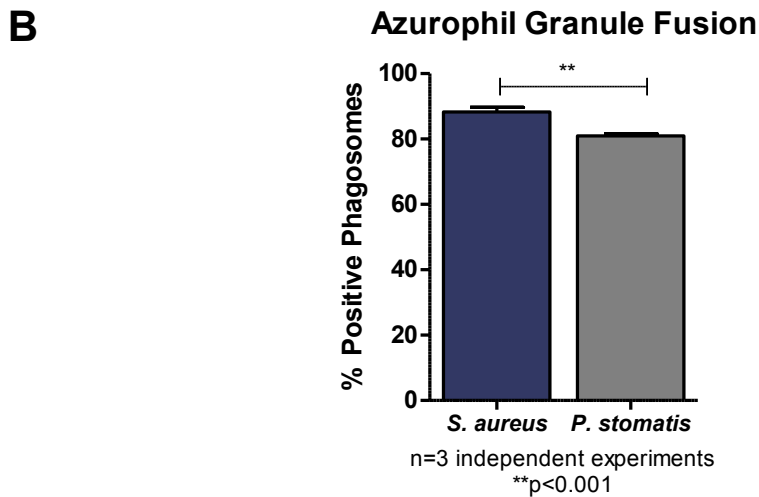
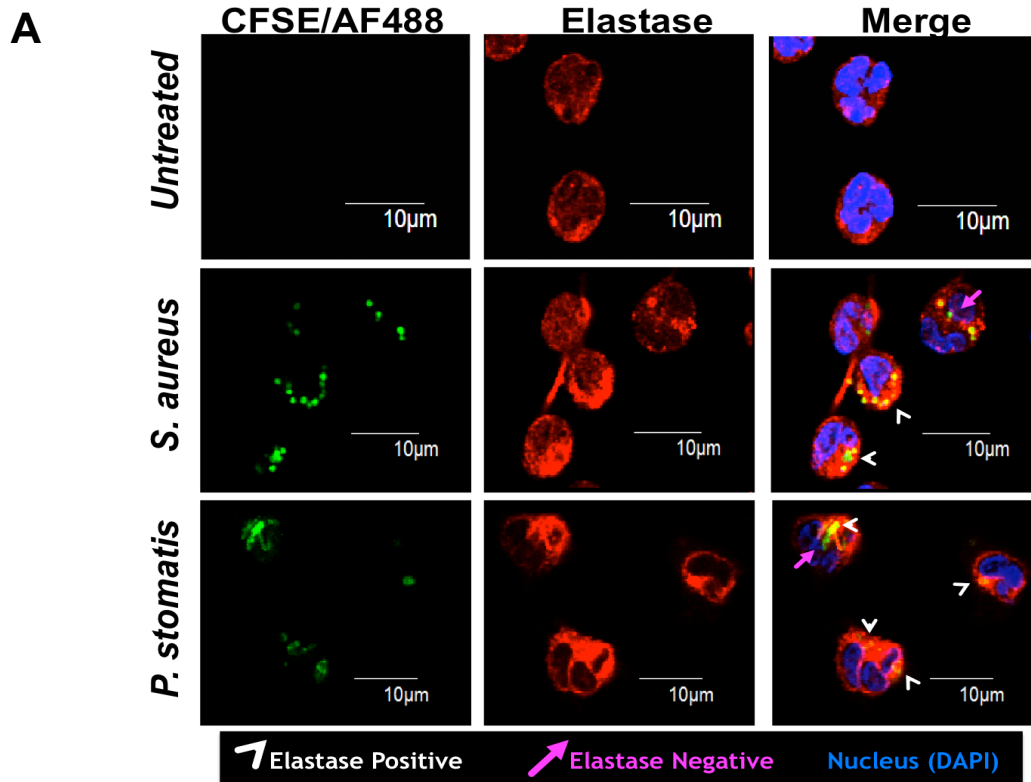
Specific and azurophil granule fusion to bacteria-containing phagosome is crucial for optimal respiratory burst responses and for phagosome maturation and efficient bacterial killing. The fusion of specific and azurophil granules with phagosomes containing *S. aureus* or *P. stomatis* was tested by immunostaining and confocal microscopy (Fig. 13A, 14A). Data expressed as a percentage of granule fusion to bacteria-containing phagosomes, indicate that there was no significant difference in specific granule fusion with the phagosomes containing

*P. stomatis* (53%) in comparison with the positive control *S. aureus* (64%), (Fig. 13B). A similar trend was observed in azurophil granule recruitment assays, where 81% of the *P. stomatis*-containing phagosomes were positive for the elastase granule marker (Fig. 14B). These results support the hypothesis that specific and azurophil granules are efficiently recruited to the *P. stomatis*-containing phagosomes, therefore promoting phagosome maturation.



**Figure 13. *P. stomatis* induces specific granule recruitment.**

Neutrophils were challenged with CFSE-labelled *P. stomatis* (MOI 10), or AlexaFluor488-labelled *S. aureus* (MOI 10) for 30 min. **(A)** Recruitment of specific granules (lactoferrin) to bacteria containing phagosomes was analyzed by immunostaining and subsequent confocal microscopy. DAPI (blue) was used to stain the cell nucleus. 100+ cells per condition were examined and phagosomes were labeled as lactoferrin positive if  $\geq 50\%$  of the phagosome was surrounded by the granule marker. White arrowheads show lactoferrin positive phagosomes and pink arrows show lactoferrin negative phagosomes. **(B)** The percentages of lactoferrin positive phagosomes demonstrate the percentage of specific granule fusion to the bacteria-containing phagosomes. Data are expressed as the mean  $\pm$  SEM of the percent of bacteria positive neutrophils from 4



**Figure 14A and 14B. *P. stomatis* induces azurophil granule recruitment.**

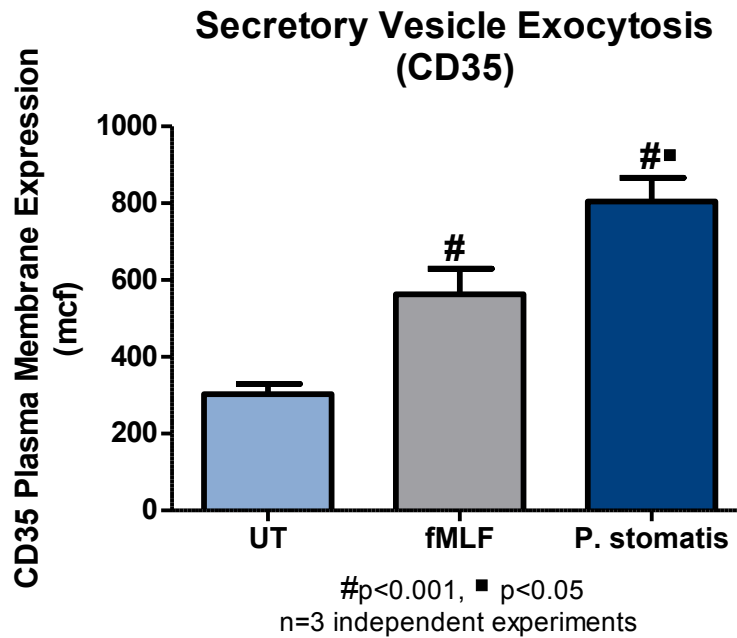
Neutrophils were challenged with CFSE-labelled *P. stomatis* (MOI 10), or AlexaFluor488-labelled *S. aureus* (MOI 10) for 30 min. **(A)** Recruitment of azurophil granules (elastase) to bacteria containing phagosomes was analyzed by immunostaining and subsequent confocal microscopy. DAPI (blue) was used to stain the cell nucleus. 100+ cells per condition were examined and phagosomes were labeled as lactoferrin positive if  $\geq 50\%$  of the phagosome was surrounded by the granule marker. White arrow heads show elastase positive phagosomes and pink arrows show elastase negative phagosomes. **(B)** The percentages of elastase positive phagosomes demonstrate the percentage of specific granule fusion to the bacteria-containing phagosomes. Data are expressed as the mean  $\pm$  SEM of the percent of bacteria positive neutrophils from 3 independent experiments. \*\*p<0.001 compared to *S. aureus*.

## Granule Exocytosis

Upon stimulation, the neutrophil granules can be mobilized towards the plasma membrane releasing their bactericidal cargo to the extracellular space. This defense mechanism contributes to priming of the respiratory burst response by increasing the expression of the NADPH oxidase membrane components (Uriarte et al., 2011). Hence, we evaluated neutrophil granule exocytosis by ELISA and flow cytometry.

Upon activation, the four neutrophil granule subtypes can undergo exocytosis, a process where they are mobilized towards the cell membrane and their cargo is released into the extracellular space. The components of the secretory vesicles and the gelatinase granules aid in early neutrophil mechanisms such as adhesion, chemotaxis and extravasation; therefore, they are the first ones to suffer exocytosis. The last granules to be released are the specific and azurophil granules, which contain the most microbicidal and cytotoxic components (Borregaard et al., 2007; Sengelov et al., 1993).

Stimulation of human neutrophils with *P. stomatis* induced significant of secretory vesicles exocytosis, measured as plasma membrane expression CD35, compared to the positive control fMLF (Fig. 15).

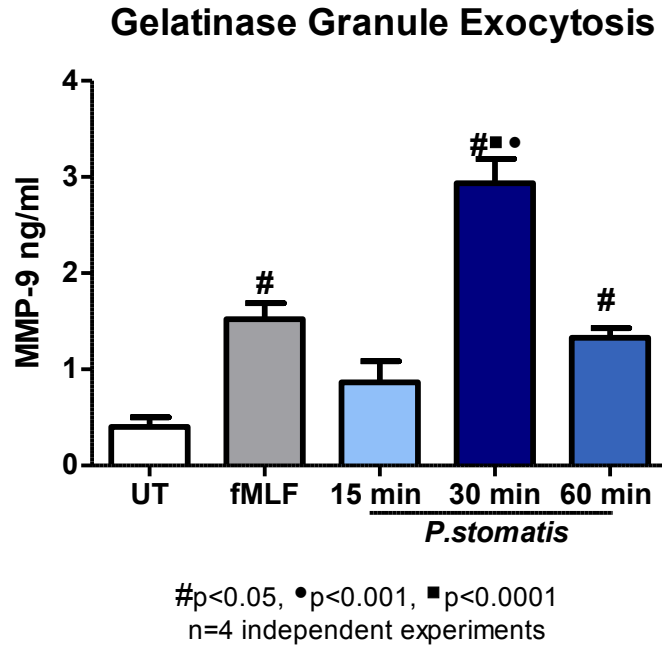


**Figure 15. *P. stomatis* induces secretory vesicle exocytosis.**

Exocytosis of secretory vesicles was analyzed by measuring the increase in cell membrane expression of the granule marker CD35. Granule exocytosis of unstimulated (UT), or fMLF stimulated (fMLF), or *P. stomatis* challenged neutrophils were tested using flow cytometry. Bacterial conditions were incubated for 30 min with an MOI of 10. Graph showing levels of secretory vesicle exocytosis. Data are expressed as mean  $\pm$  SEM of mean channel of fluorescence. #p< 0.001 compared to UT, ■p< 0.05 compared to fMLF.



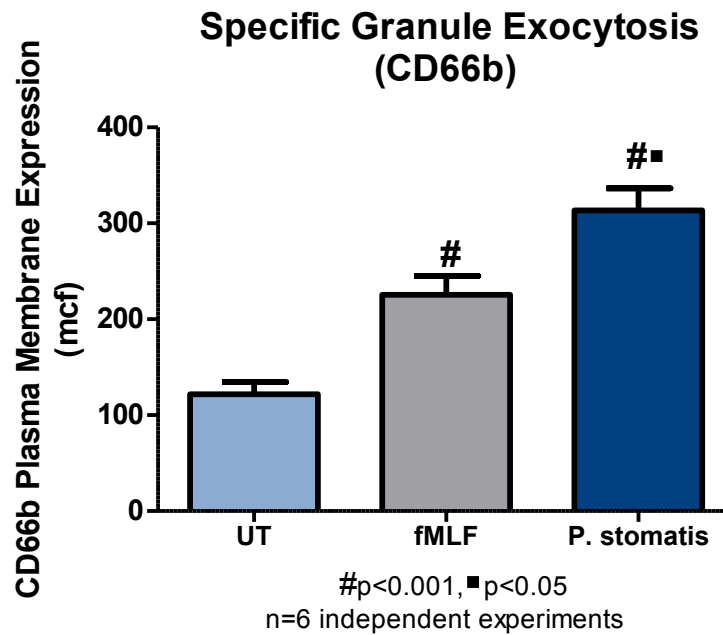
As shown in Fig 16, incubation of *P. stomatis* for 30 min with human neutrophils induced a significantly higher gelatinase granule exocytosis compared to the positive control fMLF.



**Figure 16. *P. stomatis* induces gelatinase granule exocytosis.**

Neutrophils were unstimulated (UT), or stimulated with fMLF (300 nM, 5 min), or challenged with *P. stomatis* (P.S) at a MOI of 10 for 30 min. Samples were analyzed by ELISA. Data are expressed as mean  $\pm$  SEM of MMP-9 release in ng/ml from 4 independent experiments. #p< 0.05 compared to UT, •p< 0.001 compared to *P. stomatis* at 15 and 60 min, ■p< 0.0001 compared to fMLF,.

Similarly, data presented as mean  $\pm$  SEM of the mean channel of fluorescence (mcf) show that *P. stomatis* challenge induced significant specific granule exocytosis, measured as plasma membrane expression of CD66b (Fig. 17).



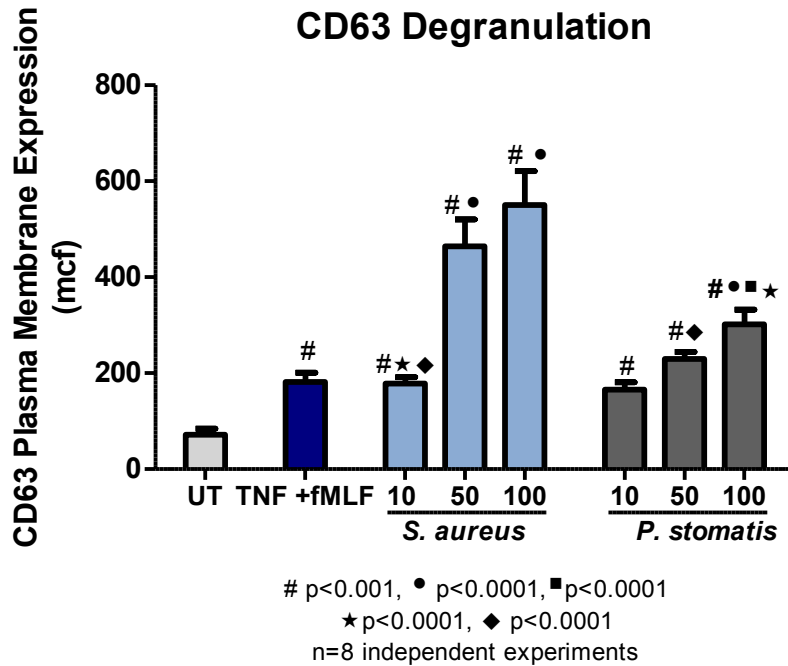
**Figure 17. *P. stomatis* induces specific granule**

Exocytosis of specific granules was analyzed by measuring the increase in cell membrane expression of the granule marker CD66b. Granule exocytosis of unstimulated (UT), or fMLF stimulated (fMLF), or *P. stomatis* challenged neutrophils were tested using flow cytometry. Bacterial conditions were incubated for 30 min with an MOI of 10. Graph showing levels of specific granule exocytosis (CD66b). Data are expressed as mean  $\pm$  SEM of mean channel of fluorescence. #p< 0.001 compared to UT, ■p< 0.05 compared to fMLF.

These results show that *P. stomatis* induces neutrophil granule exocytosis efficiently, and correlate with the hypothesis that *P. stomatis* is capable of priming and activating neutrophils readily.

Furthermore, the challenge with *P. stomatis* at MOI of 10 induced significant azurophil granule exocytosis, to the same degree as the positive controls TNF+ fMLF, or *S. aureus* (Fig. 18). To determine if the induction of

azurophil granule mobilization could be correlated to the number of bacteria per cell, cells were challenged with either *P. stomatis* or *S. aureus* at higher MOI of 50 and 100. Data from these experimental conditions show that the positive control *S. aureus* had a significant elevation of in azurophil granule exocytosis as the MOI increased from 50 to 100 (Fig. 18). Conversely, the exocytosis increase after challenge with *P. stomatis* or with the TNF+ fMLF-control at these higher MOIs was non-significant. Due to their contents (myeloperoxidase, serine proteases,  $\alpha$ -defensins, and lysozyme), azurophil granules provide the main supply for the microbicidal arsenal of neutrophils and are considered the most powerful of the granule subtypes (Amulic et al., 2012). These results further support the idea that *P. stomatis* might promote inflammation and periodontal tissue damage by the exocytosis of the highly toxic cargo contained in the azurophil granules.



**Figure 18. *P. stomatis* induces azurophil granule exocytosis.**

Neutrophils were unstimulated (UT), or stimulated with TNF- $\alpha$  + fMLF, or challenged with *P. stomatis* or with *S. aureus*. Bacterial conditions used MOI of 10, 50, or 100 for 30 min. Azurophil granule exocytosis was measured by the increase in plasma membrane expression of CD63 granule marker by flow cytometry. Data are expressed as mean  $\pm$  SEM of mean channel of fluorescence. #p< 0.0001 compared to UT, ●p< 0.0001 compared to TNF+fMLF, ■p< 0.0001 compared to *P. stomatis* MOI 10, ★p< 0.001 compared to *S. aureus*. MOI 100, ◆ p< 0.001 compared to *S. aureus*. MOI 50.

## CHAPTER 5

### DISCUSSION

Neutrophils are professional phagocytes that represent the predominant immune cell to infiltrate the gingival tissues in periodontitis patients. In periodontal diseases, they play an important role both trying to resolve infection and promoting chronic inflammation. The functional mechanisms neutrophils use to confront intruders include phagocytosis, the production of reactive oxygen species, and the release of antimicrobial molecules and neutrophil extracellular traps (NETs) (Ryder, 2010). Therefore, to better understand the role of *P. stomatis* in periodontitis, we studied its interactions with neutrophils, and found that this periodontal pathogen triggers most of the neutrophil killing mechanisms, such as generation of ROS and neutrophil granule mobilization. Nonetheless, while these responses aid in bacteria eradication, they are also harmful to the host since they promote chronic inflammation

Our CFU data indicate that neutrophils are only able to eliminate 34% of *P. stomatis* after a 30 min challenge. However CFU assays only estimate the

viability of an entire bacterial population and are unable to determine individual bacterial viability. This limitation may compromise the results if the bacteria forms aggregates or microcolonies, since that phenotype might behave differently than individual cells. Therefore, in order to assess the survival of extracellular, internalized or associated *P. stomatis* after neutrophil challenge, we utilized fluorescent dyes (BacLight assays) that indicate bacterial viability as described by Johnson & Criss (2013). Our findings from this assays show that while 80% of the extracellular bacteria remained viable, neutrophils were able to eliminate the internalized bacteria efficiently (69%) after 30 min. Moreover these results also show that after 120min neutrophils are able to eradicate the majority of the *P. stomatis* regardless of if this pathogen is internalized or not.

Previous studies state that other periodontal pathogens, such as *T. denticola* and *P. gingivalis*, are at least to some extent resistant to neutrophil phagocytosis and poorly bind to the surface of neutrophils (Ji et al., 2007; Olsen & Hajishengallis, 2016; Shin et al., 2008). Similarly, our data show that 90% of the human neutrophils in suspension failed to internalize *P. stomatis*, suggesting that neutrophils have a low phagocytic efficiency towards this periodontal pathogen.

Some bacteria utilize several different mechanisms to avoid being phagocytized by neutrophils. For example, *T. denticola*, uses a major outer sheath protein (Msp) to perturbate neutrophil actin remodeling and reorganization, relevant dynamics during phagocytosis (Amin et al., 2004; Magalhães et al., 2008; Puthengady Thomas et al., 2006).

Opsonization is an important process, in which the innate immune response against bacteria is enhanced by coating the intruders with serum proteins and/or antibodies that facilitate their internalization (Lenzo et al., 2016). Our phagocytosis results also show that there was no difference in neutrophil internalization of human serum opsonized *P. stomatis* compared to non-opsonized *P. stomatis*. However this is not the norm for other periodontal pathogens. In another study, bacterial uptake was significantly higher on phagocytes challenged with antibody opsonized *T. denticola*, or *F. nucleatum*, compared to the same non-opsonized bacterial conditions. (Shin et al., 2008). Moreover, previous studies have demonstrated that *P. gingivalis* gingipains degrade common opsonins such as IgG and the central complement component C3, avoiding the deposition of the C3b opsonin on the bacterial surface (Hajishengallis & Hajishengallis, 2014; Lenzo et al., 2016; Popadiak et al., 2007). Hence it remains to be determined if *P. stomatis* is able to avoid neutrophil phagocytosis by manipulating the complement pathways. It bears mention that due to the lack of commercial antibodies for *P. stomatis*, we were only able to test bacterial opsonization with whole human serum in this study. Besides promoting bacterial recognition and phagocytosis, antibodies also activate the neutrophils, boosting their killing responses and promoting tissue damage (Selvaraj et al., 2004; Shin et al., 2008). Since it has been shown that periodontal patients produce elevated levels of antibodies against periodontal pathogens (Ebersole, 2003), it would be interesting to assess the interactions between *P. stomatis* and neutrophils from periodontal patients.

An example of a mechanism bacteria use to evade phagocytosis, independently of host opsonization, is the downregulation of flagellar motility. It has been shown that when *P. aeruginosa* reduces motility there is a significant increase in resistance to neutrophil internalization. The authors attribute this effect to bacterial activation of the host cell PI3K/Akt signaling pathway (Lovewell et al., 2014). Since *P. stomatis* is a motile microorganism with peritrichous flagella, there is a possibility that this mechanism could be implicated on this pathogen's phagocytosis evasion.

The ability of *P. stomatis* to form long chains could be in part implicated with its defiance to phagocytosis, since the internalization of aggregated bacteria may represent a bigger challenge to neutrophils than the ingestion of single cells (Lamont & Jenkinson, 1998; Ochiai et al., 1993).

Young et al. (2006) observed that *Actinomyces* spp. attaches to the neutrophil membrane, using its type I fimbriae. This interaction not only results in phagocytosis, but it also triggers the release of lysosomal enzymes into the extracellular space contributing to the inflammation state. Similarly, during the imaging and analysis processes of this study, we noticed that *P. stomatis* is able to attach to the membrane of some of the challenged neutrophils; with the difference that this interaction does not seem to promote phagocytosis.

Neutrophils are rapidly recruited from the circulation into the periodontal pocket, where they play a key role in clearing infection. It has been suggested that some phagocytosis-resistant pathogens, such as *T. denticola*,



usually stimulate neutrophils poorly (Shin et al., 2008). However, our data show that even after avoiding internalization, *P. stomatis* still manages to activate the neutrophils and trigger most, if not all, the killing mechanism responses. This neutrophil activation might be *P. stomatis* strategy to promote chronic inflammation and its subsequent tissue damage.

Neutrophils are equipped with toll-like receptors (TLRs), in charge of the recognition of different pathogen-associated molecules, such as lipopolysaccharide (LPS), peptidoglycan, and flagellin. They play an important role in neutrophil activation (Blander & Medzhitov, 2004; Lovewell et al., 2014). Hayashi et al. (2003) determined that stimulation with LPS, lipopeptide or flagellin reduced neutrophil chemotaxis to IL-8 significantly and had a priming effect on fMLF-induced superoxide release. Hence, the activation of TLR5 receptors by *P. stomatis* flagella or TLR2 by its lipoteichoic acid and peptidoglycan, might help explain why the bacterium is able to activate the neutrophils while avoiding phagocytosis.

The induction of neutrophils' respiratory burst response with the high generation of reactive oxygen species is a potent and effective antimicrobial weapon. However, excess on ROS production affects more than just the internalized microorganism. It can also lead to periodontal tissue damage by several mechanisms, such as: DNA damage, protein damage, enzymes oxidation, proinflammatory cytokine release, and lipid peroxidation (Matthews et al., 2007; Ozmeric, 2004; Takane et al., 2002).

Our data show that *P. stomatis* challenge triggered a robust respiratory burst response in human neutrophils. A strong correlation between phagocytosis and ROS production has been suggested (Shin et al., 2008). However, while this might be true, phagocytosis is not the only mechanism to trigger an intracellular ROS response. As mentioned previously, neutrophils can produce substantial amounts of intracellular ROS also in the total absence of phagosome formation. The biological relevance of granule-localized, non-phagosomal ROS is still not clear, but they likely participate in cell signaling (Parker et al., 2014).

Additionally, we determined that *P. stomatis* is able to prime the neutrophil for an enhanced respiratory burst response after the encounter of a secondary stimuli. This is an important finding since it has been previously suggested that primed neutrophils can predispose individuals to develop periodontitis (Uriarte et al., 2016). Furthermore, it has been established that neutrophils from chronic periodontitis patients, show enhanced extracellular ROS release *in vitro* in the absence of exogenous stimulation (Matthews et al., 2007).

Neutrophil granule fusion to the bacteria-containing phagosome, and the consequential release of their cargo inside of the phagosome is important for phagosome maturation and bacterial killing. We found that 53% of the *P. stomatis*-containing phagosomes were positive for the specific granule marker lactoferrin, while 81% showed azurophil-positive (elastase) granule association. Indicating that *P. stomatis* induces neutrophil granule recruitment efficiently.

One of the limitations of this study is that since the granule

recruitment assays were analyzed by confocal microscopy, the location of the phagosomal membrane could not be determined. Therefore, it is not possible to ascertain if the intracellular *P. stomatis* is constrained inside a phagosome or if it remains in the cytoplasm. However, based on specific and azurophil granule fusion visualization — where positive phagosomes show a dense staining when integration with the phagosomal membrane occurs and negative phagosomes are characterized by a punctate staining close to the phagosome representing non-fused granules — it is feasible to infer that *P. stomatis* is internalized in neutrophil phagosomes (Allen et al., 2005; Borregaard & Cowland, 1997; Johnson & Criss, 2013b).

Our findings also indicate that *P. stomatis* induced the exocytosis of the four neutrophil granule subtypes. These in vitro results suggest that neutrophils rely on the release of their granule contents (such as lysozyme, lactoferrin, and proteolytic enzymes) to eradicate extracellular *P. stomatis* and might help explain the increase of non-viable bacteria seen after a 120 min challenge, where both intracellular and extracellular bacteria were eliminated. However, besides clearing infection, neutrophil granule exocytosis also has a negative impact on the host. Degranulation is a crucial step in neutrophil activation, and the extracellular release of the neutrophil proteases contained in the granules contributes to tissue damage and leads to the chronic inflammation state characteristic of periodontitis (Ryder, 2010; Salamone et al., 2010). Upon degranulation, the release and activation of collagenase (MMP-8) and gelatinase

(MMP-9), contained in the specific and gelatinase granules cargo, are considered crucial regulatory steps of periodontal inflammation and destruction (Shin et al., 2008). Gelatinase has been associated with gingival attachment loss and mean tooth mobility (Teng et al., 1992). Similarly, the extracellular release of elastase, a component contained in the azurophil granule subtype, can hydrolyze extracellular matrix proteins such as elastin, fibronectin, and collagen types III and IV and has been strongly associated with periodontal diseases (Kantarci et al., 2003; Shin et al., 2008). In contrast, the extracellular superoxide dismutase, usually stored in secretory vesicles, is an antioxidant enzyme that provides protection against oxidative degradation of matrix components. Iversen et al. (2016) determined that when this enzyme is released into the extracellular space it may offer protection against the reactive oxygen species generated as a result of respiratory burst activity of activated neutrophils. Hence we suggest that the granule exocytosis and the oxidative burst response enhancement by *P. stomatis*, might be a bacterial strategy to further activate/stress the neutrophil and to promote inflammation and the breakup periodontal tissue that might be beneficial to other periodontal pathogens.

Finally, as periodontitis is of polymicrobial nature, it is reasonable to consider that *P. stomatis* does not exist exclusively as planktonic cells in the periodontal pocket; rather it forms part of a multi-species biofilm. Hence it may be phenotypically distinct from the in vitro cultures analyzed in this study. As part of a biofilm, bacteria have different properties compared to their planktonic state,

such as differential gene expression, and enhanced resistance to antimicrobials and the host immune defense (Cerca et al., 2006; Domenech et al., 2013; Hogan & Kolter, 2002; Resch et al., 2005). For example, it has been established that *S. aureus* genes responsible for urease activity and the response to oxidative stress are up-regulated in a biofilm environment, while genes responsible for toxins and proteases were up-regulated under planktonic growth conditions (Resch et al., 2005). Furthermore, another study showed that even though neutrophils are able to penetrate *S. aureus* biofilms, they cannot internalize the bacteria efficiently (Leid et al., 2002). Therefore further studies are needed to better understand the role *P. stomatis* plays within the context of periodontal biofilm communities.

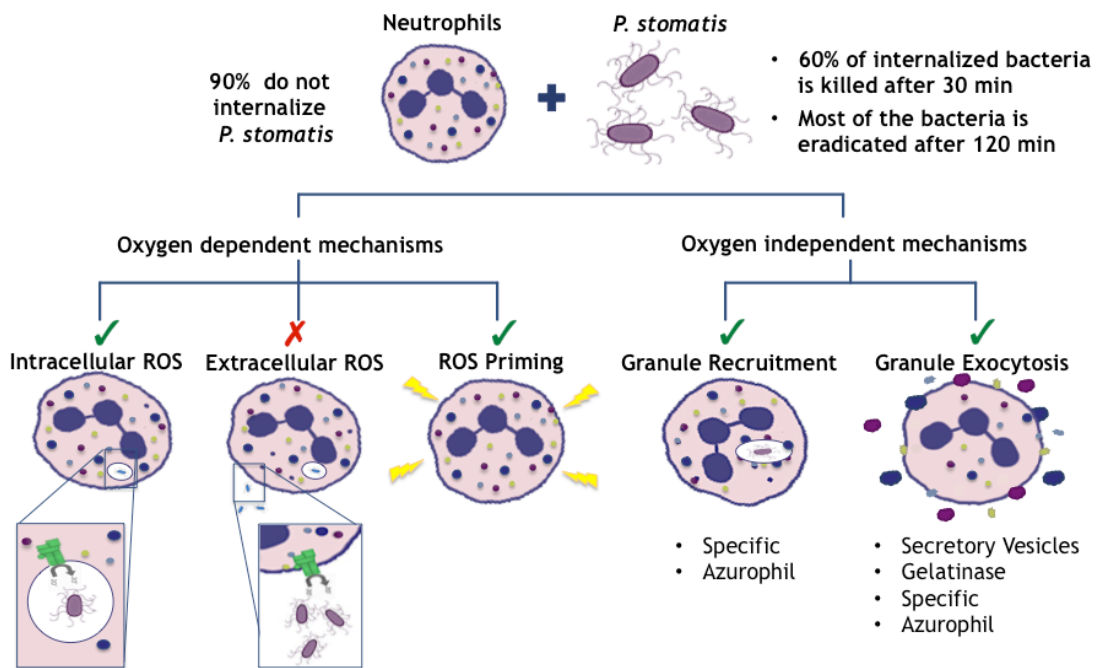
## CHAPTER 6

### SUMMARY AND CONCLUSION

In conclusion, these results suggest that although 90% of neutrophils do not internalize *P. stomatis* efficiently, they still get activated and mount their killing responses against this pathogen (Fig. 18). After *P. stomatis* challenge for 30 min, neutrophils were able to mount a robust intracellular respiratory burst response and efficiently recruit specific and azurophil granules to the bacteria-containing phagosomes; eradicating 60% of the internalized bacteria. Moreover, *P. stomatis* was able to prime the neutrophils and modulate the respiratory burst response to other stimuli.

Similarly, as a result of *P. stomatis* stimulation, human neutrophils undergo exocytosis of their four granule subtypes. We speculate that the release of the different neutrophil granule contents, such as lysozyme, gelatinase, collagenase and elastase, are responsible for the bacterial killing of *P. stomatis* seen after a 120 min challenge, which was independent of bacterial

internalization. However these neutrophil responses go beyond bacteria eradication, they also promote the chronic inflammation and tissue destruction that characterize periodontal diseases. These data demonstrate previously unexplored aspects of this newly appreciated taxon and how these organisms modulate neutrophil function.



**Figure 19. Model of the interactions between *P. stomatis* and neutrophils.**

Neutrophils had a low phagocytic efficiency for *P. stomatis* compared to *S. aureus*. Around 60 % of the internalized bacteria was killed within a 30 min interaction with human neutrophils. *P. stomatis* challenge induced a robust respiratory burst response. Bacteria stimulation resulted in minimal induction of superoxide release by neutrophils. Neutrophils were able to efficiently recruit specific and azurophil granules to *P. stomatis*-containing phagosomes. *P. stomatis* induced significant exocytosis of the four neutrophil granules.

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## CURRICULUM VITA

Emeri Jimenez Flores

### Education

University of Louisville School of Dentistry, KY, USA Masters of Science in Oral Biology Candidate (M.S)	August 2016
Advanced Technology Institute (ITAV), N.L, México Advanced Education in Laser Dentistry	October 2013
Universidad Autónoma de Nuevo León Faculty of Dentistry, México Graduated with Highest Honors, Doctor of Dental Surgery (D.D.S)	July 2013

### Awards, Scholarships and Honors

2nd Place Dental Student Award at <i>Research! Louisville</i>	October 2015
Graduate Program in Oral Biology Tuition Scholarship Award	August 2015
University of Louisville Summer Research Award	May 2015
Mexican National Council for Science and Technology Tuition Award	August 2014- 2016
Academic Excellence Award in 2013 Graduating Class	July 2013

### Leadership positions

President, University of Louisville School of Dentistry Student Research Group	January- December 2015
Coordinator, CONL (Nuevo Leon Dental Association)	January 2014 – July 2014
Member of the Board, AMO (Women Dental Association)	January 2014 – July 2014
Member of the Board as U.A.N.L. Representative, CONL	January –December 2013
President, CONL Student Division	January 2012 – December 2012

## Publications

Ozone Therapy Benefits In Dental Practice. Jiménez Flores E.; Sánchez Nájera R. I., Solís Soto J.M., Nakagoshi Cepeda A.A., Nakagoshi Cepeda S.E. RESPYN 2011: Ed. Esp.3: S2.7

Portland Cement Presents Similar Biocompatibility As Traditional Endodontic Sealers. Sánchez Nájera R.I., Solís Soto J.M., Nakagoshi Cepeda A., Jiménez Flores E., Rodríguez Delgado I.(2011).SSN 2007-3291

## Presentations And Seminars

Research!Louisville, Louisville, KY October 2015  
Poster Presentation: "*Peptoanaerobacter Stomatidis* interactions with neutrophils."

Greater New York Dental Meeting. New York, NY December 2013  
Poster Presentation: "Gorlin Goltz: A clinical case report."

Greater New York Dental Meeting. New York, NY December 2013  
Poster Presentation: "Polymeric Nanoparticles as Vehicles of plant-derived excipients."

iTAV Laser in Dentistry Fellowship, III module October 2013  
Seminar: " Dental Ozone and photodynamic therapy. "