ORAL POLYMICROBIAL COMMUNITY ACTIVATION OF TOLL-LIKE RECEPTOR 4

Thao Thanh To

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Reading Committee:

Richard P. Darveau, Chair
Frank A. Roberts
Jeffrey S. McLean

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ABSTRACT

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Thao Thanh To

Chair of the Supervisory Committee:
Professor Richard P. Darveau
Department of Periodontics

Chronic periodontitis is characterized by not only the dysbiotic polymicrobial community which lies below the gumline, but also how this community triggers overwhelming and persistent inflammatory responses from the host innate immune system. In this work, we describe how the subgingival microbial community as a whole engages a major innate immune receptor, Toll-like receptor 4 (TLR4), in either health or disease states. The plaque polymicrobial community in disease potently activates TLR4; however, subgingival plaque collected from healthy sites not only suppress NF-kB signaling through TLR4 suppression, but also dampens expression of neutrophil recruitment molecule, E-selectin, on the surface of endothelial cells. To our knowledge, this is the first report of any clinical patient sample of a polymicrobial community acting on TLR4 in an antagonistic

manner. Additionally, we demonstrate how the metabolic relationship between two bacteria, highly associated together in disease- *F. nucleatum* and *P. gingivalis*- synergistically work together to promote a pro-inflammatory state through TLR4 activation. This phenomena is not observed when *F. nucleatum* is grown in co-culture with *S. gordonii*, a relationship more associated with a more healthy periodontal status. Taken together, our work elucidates the role of polymicrobial communities on TLR4 activation in health and disease.

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GLOSSARY

amu	atomic mass unit
BLAST	Basic Local Alignment Search Tool
BOP	bleeding on probing
CAL	clinical attachment level
CD14	cluster domain 14
ermF-AM	erythromycin / clindamycin resistance gene
E-selectin	endothelial selectin
FN0814	putative propionate CoA transferase gene in *F. nucleatum* ATCC 25586
FN0815	putative propionate permease gene in *F. nucleatum* ATCC 25586, *prpP*
FnLPS	*F. nucleatum* ATCC 25586 LPS
HEK293	human embryonic kidney 293 tissue cells
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MD-2	myeloid differentiation protein-2; TLR4 co-receptor
m/z	mass-to-charge ratio
NF-κβ	Nuclear factor-kappa beta
Pam3CSK4	synthetic triacylated lipopeptide; TLR2 agonist
PD	probing depth
Pg1435LPS	*P. gingivalis* tetraacylated, monophosphorylated LPS; potent TLR4 antagonist

PI	plaque index
prpP	propionate permease
qPCR	quantitative polymerase chain reaction
subsp	subspecies
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TYHK	bacterial growth medium consisting of trypticase soy, yeast extract, hemin and vitamin K

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DEDICATION

To my family here in the United States and those still in Vietnam. I am very blessed with such a large, loving, and supportive family.

To my parents, Chot To and Hue Ha, who worked tirelessly to ensure that every opportunity was a possibility for me. They inspire me to work hard and love life every day.

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CHAPTER I:
INTRODUCTION

"I am in the habit of rubbing my teeth with salt in the morning, and then rinsing my mouth with water; and often after eating, to clean my back teeth with a toothpick, as well as rubbing them hard with a cloth, wherefore my teeth back and front remain as clean and white that only a few people of my age (fifty-one) can compare with me. Also when I rub my gums with hard salt, they will not bleed. Yet all this does not make my teeth so clean but that I can see, looking at them with a hollow mirror, that something will stick and grow between some of the molars and teeth, a little white matter, as batter. Observing it I judged that although I could not see anything moving in it there were yet living animalcules in it. I then mixed it several times with pure rain-water, in which there were no animalcules and also with saliva that I took from my mouth after eliminating the air bubbles lest these should stir the spittle. I then again and again saw that there were many small living animalcules in the said matter, which moved very prettily."

-Antonie van Leeuwenhoek

DENTAL PLAQUE IS A POLYMICROBIAL COMMUNITY AND ELICITS HOST INNATE IMMUNE RESPONSES

In 1676, Antonie van Leeuwenhoek detailed in his notebook about diverse microorganisms residing in material scraped from his own teeth, opening the doors for not only the study of bacteriology, but also modern oral microbiology (Dobell, 1932). It is remarkable that he also recognized even after rudimentary cleaning, a "batter" would "stick and grow" and mature on and between the tooth surface. This substance is what we recognize today as one of the most well characterized polymicrobial communities residing within the human body.

It is estimated that the human oral cavity is colonized by over 700 different species of bacteria and that over half are yet to be cultured and characterized (Dewhirst *et al.*, 2010).

During the shift from periodontal health to disease, the number of bacteria increases and composition of the dental plaque biofilm changes from predominantly aerobic and gram – positive species to largely anaerobic and gram-negative flora (Socransky *et al.*, 1998, Marsh, 2003). Ecological studies based on the detection of 40 different species in over 10, 000 plaque samples have defined five major bacterial complexes, each consisting of bacterial species that are found in strong association with one another in plaque (Socransky *et al.*, 1988, Socransky et al., 1998). The highly ordered succession of bacteria in the plaque biofilm results in a unique polymicrobial community, benefitting its members more so than living on their own. Examples of such advantages in this community include 1) a broader habitat range for growth, 2) a more efficient metabolism, 3) an increased resistance to stress and antimicrobial agents, and 4) an enhanced virulence (Marsh, 2005). The perks of community-living allow the dental plaque polymicrobial community to embed itself in extracellular matrix and subsist on the surface of the tooth surface as a biofilm (Socransky & Haffajee, 2002, Marsh, 2005), further allowing the community to directly interact with its host. These microbial community - host interactions can change when the microbiota itself changes from a commensal role to a pathogenic one in response to a trigger or ecological perturbations, contributing to the etiology of periodontal disease. This is the basis of the ecological plaque hypothesis (Marsh, 1994), which emphasizes the contribution of a mixed microbiota rather than of an individual species to development of periodontal disease.

Despite this emphasis on the microbial community, the response from the host is equally significant for pathology in the disease. Characteristics of periodontitis include

pronounced inflammation, tissue destruction, and alveolar bone resorption, which are all mainly a result of an aggravated attempt by the host to destroy or control the microbial plaque infection (Page *et al.*, 1997). Increases in pro-inflammatory cytokines, such as IL-1β and TNF, as a response to infection, may disrupt the balance of bone remodeling, skewing the proportion of RANKL, or receptor–activator of nuclear factor-κB ligand, to OPG, or osteoprotegrin (Darveau, 2010, Nagasawa *et al.*, 2007). Additionally, activation of transcription NF-kB through innate immune recognition of bacterial components results in the induction of chemokine, IL-8, which directs migration of neutrophils to the site of infection. Hyper-responsive neutrophils enact their phagocytic functions, resulting in soft tissue destruction. However, inability to summon neutrophils into the periodontium as a result of "local chemokine paralysis" or because of generally low levels of neutrophils in the host, like in neutropenia, can also allow evasion from the host and persistence of the pathogenic microbial community (Darveau, 2010, Ye *et al.*, 2011).

TOLL-LIKE RECEPTOR 4: SENTRY DETECTION OF GRAM-NEGATIVE INFECTIONS

Toll-like receptors (TLRs) comprise an evolutionarily conserved family of pathogen recognition receptors, or PRRs, of which 12 have been described and are widely expressed on a variety of innate immune cells, including dendritic cells, macrophages, mast cells, neutrophils, and endothelial cells. These TLRs have broad specificity for conserved molecular structures of bacteria (Janeway & Medzhitov, 2002, Medzhitov & Janeway, 2000, Medzhitov, 2007). Activation through binding of the TLR results in the cell initiating

effector functions important for innate immune clearance of bacterial infections. Specifically, the lipid A moiety of lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is recognized by the TLR4 complex. However, the lipid A must first interact with other adaptor molecules before engaging TLR4, specifically binding to LPS-binding protein (LBP), which then loads the bound lipid A onto a glycosylphosphatidyl-tethered peripheral membrane protein CD14. The lipid A is then transferred to the TLR4-MD2 heterodimer and this triggers a conformational change in the TLR4 complex to activate signaling (Kim *et al.*, 2007, Park *et al.*, 2009). Downstream intracellular signaling events through adaptor protein MyD88 subsequently result in the activation of the transcription factor, nuclear factor kappa-beta (NF-kB). NF-kB activation induces transcription of many pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and chemokine IL-8, which attracts the phagocytic cells, neutrophils, to the site of infection (Lawrence, 2009). However, dysregulation of expression levels of any of these mediators – either too high or too low – can result in excessive inflammation from the host or devastating infection from the pathogens (Jain & Darveau, 2010, Dinarello, 2000).

The expression of TLR4 in gingival tissue has been evaluated in both healthy and diseased periodontal tissue. Healthy gingival tissue has been shown to express low levels of TLR4 in epithelial layers and connective tissue, likely present for critical immune surveillance during periodontal health. Evidence suggests that TLR4 is mostly expressed on the surface of dendritic cells and macrophages; however, the cells expressing this receptor have not been full characterized (Ren *et al.*, 2005, Mori *et al.*, 2003). Conversely, gingival tissue expresses

significantly higher levels of TLR4 in tissues collected from patients with chronic periodontitis (Beklen *et al.*, 2008, Mori et al., 2003, Muthukuru *et al.*, 2005). Additionally, patients who carry Asp299Gly and Thr399Ile polymorphisms in the TLR4 gene may be at higher risk for bacterial infections, including periodontal disease (Ozturk & Vieira, 2009, Schröder *et al.*, 2005). Although there is recent debate on the strength of association between periodontal diseases and these specific single-nucleotide polymorphisms in TLR4 (Folwaczny *et al.*, 2004), disruption or dysregulation of TLR4 signaling is highly relevant for persistence of the polymicrobial community in disease.

LIPID A STRUCTURAL MODIFICATIONS IN RESPONSE TO ENVIRONMENT RESULTS IN DIVERSE TLR4 ACTIVATION

Gram-negative bacteria, like *Escherichia coli, P. gingivalis* and *F. nucleatum*, have two distinct membranes: an inner membrane and an outer membrane, of which lipopolysaccharides (LPS) are a major component. LPS is composed of three parts: core polysaccharides, O-antigen repeats and lipid A, which anchors the entire molecule to the outer leaflet of the outer membrane (Raetz *et al.*, 2009, Wang & Quinn, 2010). Consequently, the lipid A portion is highly hydrophobic and the chemical structure has been most heavily studied in *E. coli*. The lipid A of *E. coli* is composed of two β,1-6 linked glucosamine sugars, which are bisphosphorylated at the 1- and 4'-position. Two myristoyl fatty acid residues are ester- linked at 3- and 3'- positions and two are amide-linked at the 2- and 2'-positions. These are subsequently acylated at the 3-hydroxy position by lauric and myristic acid residues, respectively (Raetz, 1990). This unique lipid is also termed "endotoxin" for its toxic

and pyrogenic properties. However, as detailed above, detection of this molecule through TLR4 is critical for clearance of infection in health (Beutler & Rietschel, 2003).

Consequently, modulation of the inflammatory response through TLR4 can be appropriated by some bacteria, which can alter their lipid A composition and structure in response to changes in environmental conditions. These evolved mechanisms have been of interest in the study of many Gram-negative bacteria in the recent years. Alterations in the structure of the canonical lipid A structure described previously can alter host activation of TLR4. For instance, *Yersinia pestis*, the causative agent of plaque, can cause infection in humans through a flea bite. Inside the body temperature of the flea at 21 – 27 °C, *Y. pestis* will synthesize six fatty acid chains on the lipid A and activates, or **agonizes**, an immune response through TLR4. However, in the body temperature of humans at 37 °C, the lipid A is only composed of four fatty acid chains, which suppresses TLR4 activation and allows the bacterium to evade detection by the immune system through TLR4 **antagonism** (Montminy *et al.*, 2006). Other bacteria can additionally alter the lipid A structure by modifying the phosphate groups. Oral pathogen, *Porphyromonas gingivalis* can remove the 1- and 4'-phosphate residues with enzymes, LpxE and LpxF, respectively, resulting in a heterogeneous mixture of lipid A molecules (Coats *et al.*, 2009a, Coats *et al.*, 2009b). For example, under low-hemin conditions, *P. gingivalis* synthesizes primarily non-phosphorylated lipid A. In higher hemin conditions representing the increased vascular ulceration in gingival tissues during periodontal disease, the bacterium produces primarily a mono-phosphorylated, tetra-acylated lipid A species (Curtis *et al.*, 2011, Al-Qutub *et al.*, 2006). Since both phosphate

groups interact with TLR4 and MD2 to directly to elicit the conformational change necessary for downstream signaling, loss of one of the phosphate groups in addition to loss of acyl chains or loss of both phosphate groups results in an immune-evasive or immune-suppressive phenotype, respectively (Curtis et al., 2011, Kim et al., 2007, Park et al., 2009).

FUSOBACTERIUM NUCLEATUM: ROLE IN PERIODONTAL DISEASE AND LIPID A COMPOSITION

F. nucleatum is a Gram-negative obligate anaerobe with a long tapered rod, or fusiform, shape. It is found ubiquitously in the gingival plaque, regardless of periodontal health or disease status. Additionally, its numbers appear to increase with severity of disease (Kolenbrander, 2000, Kolenbrander *et al.*, 2006, Socransky et al., 1988, Haffajee *et al.*, 2006). This bacterium plays a critical role in the development of the oral microbial community in periodontal disease. It is the only bacterium able to co-aggregate with both early and late colonizers described previously (Kolenbrander & London, 1993, Kolenbrander et al., 2006), serving as critical bridging organisms in the microbial community (Figure 1). Co-aggregation with *F. nucleatum* allows even strict anaerobes, such as *P. gingivalis*, to survive planktonically in aerated and CO_2-depleted conditions (Bradshaw *et al.*, 1998, Diaz *et al.*, 2002). Furthermore, if *Tannerella forsythia* or *Prevotella intermedia* is present in plaque, other anaerobes such as *F. nucleatum* are usually also present (Yoshida *et al.*, 2005, Socransky et al., 1998, Suzuki *et al.*, 2004, Ali *et al.*, 1994). Taken together, this suggests that this bacterium provides protection to pathogenic, obligate anaerobes by forming mixed species aggregates with commensal, aerobic bacteria (Bradshaw et al., 1998).

Another key feature of *F. nucleatum* is its ability to invade host cells. In fact, the organism has been shown to adhere to and invade fibroblasts and epithelial and endothelial cells (Han *et al.*, 2000, Dabija-Wolter *et al.*, 2009). Additionally, invasion of gingival fibroblasts was found necessary for *F. nucleatum* activation of NF-kB through a RIG-I (Lee & Tan, 2014). Invasion of oral epithelial cells and aortic endothelial cells by *P. gingivalis* is also enhanced by *F. nucleatum* in co-culture (Saito *et al.*, 2008), demonstrating the synergistic virulence of *F. nucleatum* co-aggregation with pathogens.

Outside of the periodontium, *F. nucleatum* is commonly found in other sites in the body and in other chronic infections, including the human gut, placenta, liver, joints, lungs, and blood (Han & Wang, 2013, Allen-Vercoe *et al.*, 2011, Han *et al.*, 2004). Lastly, *F. nucleatum* was found to induce murine fetal death through a TLR4-specific pathway, indicating that this bacteria is able to avoid detection by the immune system very well and that this evasion, like in other bacteria, may be regulated in part by lipid A modifications and its TLR4 interactions (Liu *et al.*, 2007). Consequently, further studies must be pursued to understand the interaction of possible *F. nucleatum* lipid A alterations and TLR4 activation.

Only two previous studies have attempted to structurally characterize the lipid A of *F. nucleatum* (Asai *et al.*, 2007, Hase *et al.*, 1977). However, these analyses focused on one moiety only and did not evaluate the diversity of lipid A present. The lipid A structure derived from these studies conclude the general structure is bis-phosphorylated and hexaacylated similarly to canonical *E. coli* lipid A. Unlike that of *E. coli*, *F. nucleatum* lipid A has a higher molecular weight and longer fatty acid groups, composed of C14 and C16

chains (Figure 2) (Hase et al., 1977, Asai et al., 2007). Constrastingly, analysis of the *F. nucleatum* ATCC 25586 genome did not identify ORFs for enzymes similar to *E. coli lpxL* (myristoyl or C14 acyltransferase) and *lpxM* (palmitoyl or C16 acyltransferase) enzymes (Kapatral *et al.*, 2002). The conflicting studies of this bacterium's lipid A prompt further analysis to determine if like other pathogens, *F. nucleatum* can alter its lipid A composition and its relationship with the host through TLR4.

HYPOTHESIS AND SPECIFIC AIMS

Our overall hypothesis is that specific microbial interactions in a subgingival microbial community contribute to lipid A alterations which affect TLR4 activation in the host. The overall aim of this project is to characterize how the subgingival plaque microbial community interacts with TLR4 in periodontal health or disease. Specifically, we determined to what extent subgingival plaque interacts with TLR4 and identify how specific metabolic exchanges between certain bacteria affect TLR4 activation.

Specific Aims

Specific Aim 1. Functional characterization of *ex vivo* microbial community sample, subgingival plaque.

The first Aim examined the ability of *ex vivo* subgingival plaque samples to engage TLR4 in an in vitro assay and identify TLR4 antagonistic behavior.

Specific Aim 2. Biochemical characterization of *F. nucleatum* lipid A heterogeneity in a microbial community.

Focusing on a major member of the subgingival microbial community, Aim 2 will examine the interactions between *F. nucleatum* and other oral bacteria in health or disease and how these interactions affect *F. nucleatum* lipid A composition.

Specific Aim 3. Genetic mechanisms contributing to *F. nucleatum* lipid A heterogeneity. Finally, Aim 3 will determine the genetic contributions to lipid A heterogeneity in *F. nucleatum*. Specifically, by identifying and deleting a propionate transporter in *F. nucleatum* ATCC 25586

The work described here demonstrates the overall capacity of a subgingival polymicrobial community to not only activate TLR4, but also dampen signaling through this pathway. This work further describes how one member of this microbial community, *F. nucleatum*, interacts with its coaggregating microbial neighbors and their byproducts to modify lipid A structure and how these changes affect TLR4 activation.

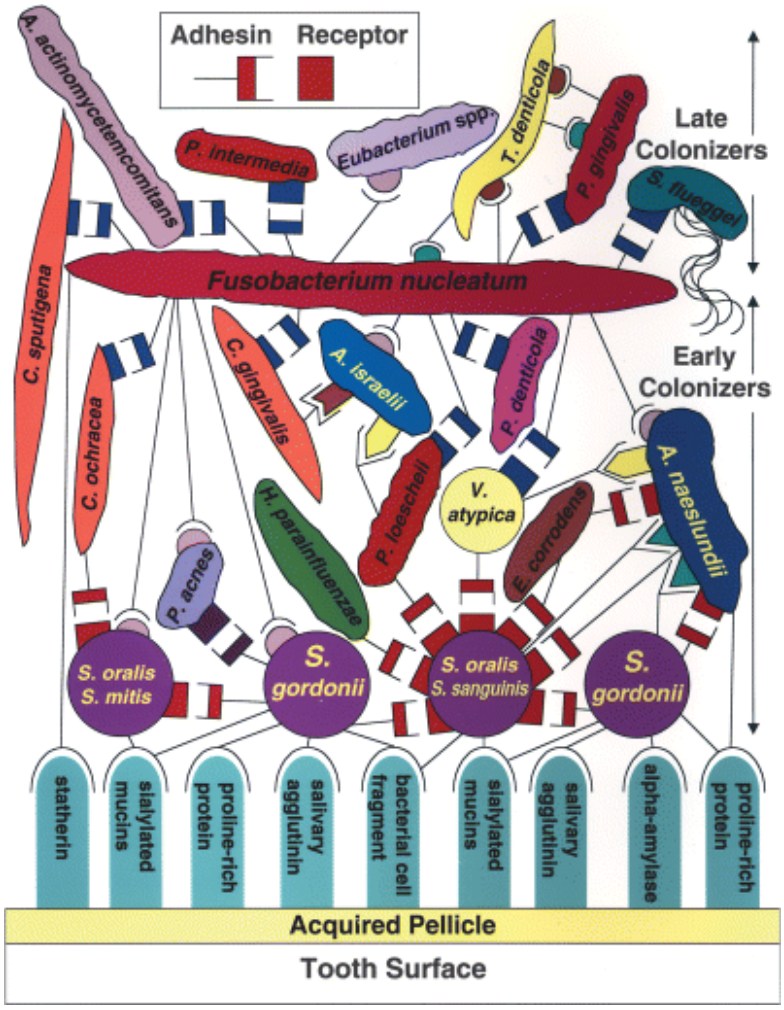
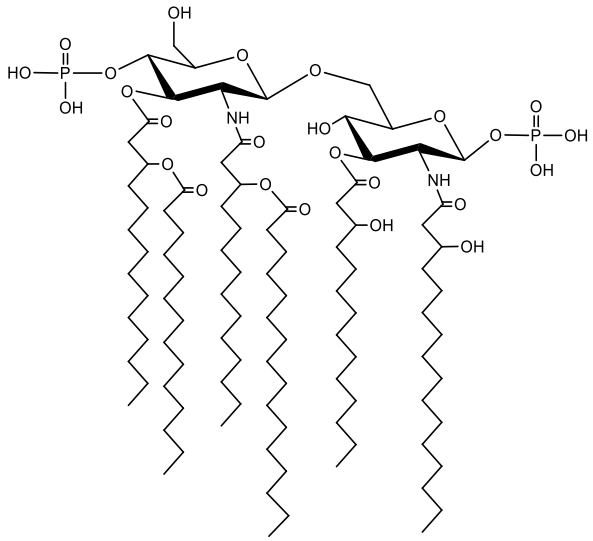


Figure 1. Spatiotemporal model of oral bacterial colonization, demonstrating the unique role of *Fusobacterium nucleatum* in the maturation of the microbial community structure. This bacterium serves as a "bridging" organism between early late colonizers ((Kolenbrander et al., 2006).

A



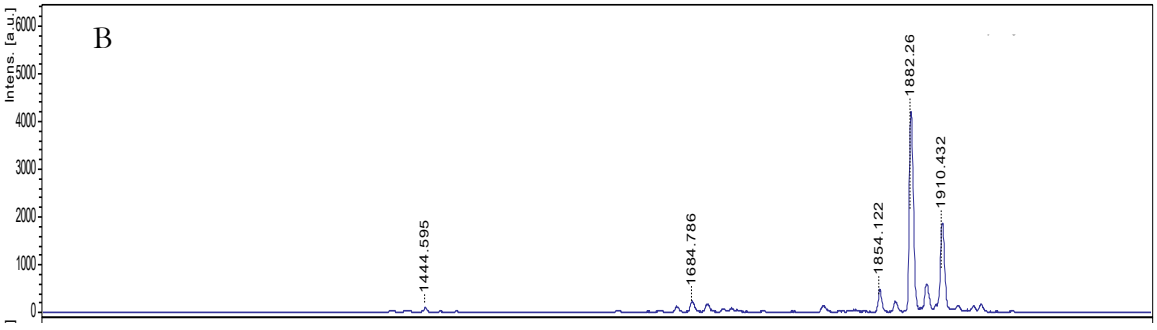


Figure 2. Suggested structure of *F. nucleatum* lipid A derived from negative mode MALDI-TOF/TOF precursor m/z 1882 (A). Negative-ion mode MALDI-TOF mass spectrum of lipid A extracted by Caroff hydrolysis of purified F. nucleatum lipopolysaccharide (B)

CHAPTER II:
SUBGINGIVAL PLAQUE ACTIVATION AND ANTAGONISM OF INNATE IMMUNE SYSTEM

INTRODUCTION

Periodontal disease is characterized by marked inflammation and destruction of bone and gingival tissue. Although the disease can be classified into different subtypes (Armitage, 1996), bacterially-induced periodontitis in adults is often a chronic inflammatory condition in which pathogenic plaque biofilm accumulates and adheres to the tooth surface above and below the gingiva. These supra- and sub-gingival plaque biofilms not only differ in location, but also in microbial composition and in relation to the development of periodontal diseases (Ximénez-Fyvie *et al.*, 2000). Although suspected periodontal pathogens may be detected in supra-gingival plaque from diseased sites, the biofilm below the gingiva ultimately interacts with the periodontium and resides in a distinct environment, limited by space and host immune protection but enriched with nutrients from gingival crevicular fluid (Darveau *et al.*, 1997).

Consequently, the sub-gingival plaque biofilm also includes bacterial antigens, which directly engage the innate immune system at the site of infection. One of these antigens, lipopolysaccharide or LPS, is a well-characterized ligand specific to innate immune receptor, Toll-like receptor 4 (TLR4). LPS is located in the outer membrane of gram-negative bacteria and structural differences can potentiate different activities on TLR4 signaling (Berezow *et al.*, 2009, Coats *et al.*, 2011). For example, *Fusobacterium nucleatum* LPS can potentiate a

relatively strong TLR4 agonistic response due to its bisphophorylated, hexaacylated lipid A moiety, the endotoxic portion of LPS which interacts directly with the TLR4 signaling complex (Lappin *et al.*, 2011). On the other hand, other periodontal bacteria, such as *Porphyromonas gingivalis* may modulate its LPS structural composition by removing phosphate residues and acyl chains on its lipid A backbone. These LPS structures antagonize TLR4 activation when mixed with strong agonist *E. coli* LPS (Coats *et al.*, 2003). Furthermore, the gram positive bacterial cell wall component, lipoteichoic acid, a known TLR2 activator, can also act as a TLR4 antagonist by interacting with co-receptor CD14 (Sugawara *et al.*, 1999). Therefore, the sub-gingival oral microbial community has the potential to modulate TLR4 activity by the relative expression of TLR4 agonists and antagonists. In addition, the modulation of TLR4 activity is also dependent on the expression levels of TLR4 and MD-2 (Coats *et al.*, 2005). Consequently, the potential for modulation of TLR4 activity as a component of periodontal homeostasis (Darveau, 2010) exists both from the sub-gingival microbial community as well as from the host as manifested in the expression levels of key TLR4 activation pathway components found in the local periodontal environment (Ren et al., 2005).

Therefore, in this study, TLR4 activation as well as inhibition was determined for sub-gingival plaque samples obtained from clinically healthy and diseased sites where both the microbial composition and expression of TLR4 pathway components are known to be altered (Ren et al., 2005). In addition, TLR2 activation was examined to determine if periodontal health status affected activation of this key inflammatory mediator. It was

found, consistent with the inflammatory nature of periodontitis, that diseased plaque samples potently activated both TLR2 and TLR4 and that these activities were associated with increasing disease. These data demonstrate a strong pro-inflammatory state in response to a dysbiotic microbial community in disease. In contrast, plaque sampled from healthy sites exhibited both TLR4 activation and antagonism. TLR4 antagonism from human clinical samples is novel and suggests that TLR4 modulation may contribute to periodontal health homeostatic mechanisms.

MATERIALS AND METHODS

Study population

Systemically healthy, untreated patients (9 males and 6 females; age range: 43 to 61 years) with generalized chronic periodontitis were recruited in this study while seeking dental treatment in the School of Dentistry, Ege University, İzmir, Turkey. The study was conducted in full accordance with ethical principles, including the World Medical Association's Declaration of Helsinki, as revised in 2000. The study protocol was explained, and written informed consent was received from each individual before clinical periodontal examinations and subgingival plaque sampling. Medical and dental histories were obtained and smoking habits were recorded. Individuals with medical disorders, such as diabetes mellitus, immunological disorders and those who had antibiotic or periodontal treatment in the last 6 months were excluded from the study.

Individuals with chronic periodontitis were diagnosed in accordance with the clinical criteria stated in the consensus report of the World Workshop in Periodontitis (Armitage, 1996). These individuals had ≥ 4 teeth in each jaw with a probing depth (PD) of ≥5 mm, clinical attachment level (CAL) of ≥ 4 mm, and ≥ 50% alveolar bone loss at least in two quadrants. Assessment of the extent and severity of alveolar bone loss was done radiographically. Bitewing radiographs were evaluated for interproximal bone loss from the cemento-enamel junction of the tooth to the bone crest. These individuals also had bleeding on probing (BOP) at > 80% of the proximal sites.

Subgingival plaque sample collection

For the diseased samples, the deepest 3 pockets were selected and pooled. Supragingival plaque was first removed from the sample teeth with sterilized Gracey curettes and gauze. The site was then cleaned and isolated using cotton roles and air dried gently. Another sterilized Gracey curette was inserted to the deepest part of the pocket and removed applying a slight force towards the root surface. The tip of the curette was then inserted in the microfuge tube containing 0.5 ml distilled water and shaken until the plaque was removed from the curette.

For the healthy subgingival plaque samples, in the same patient 3 healthy sites that PD < 3 mm and did not show any sign of inflammation and bleeding on probing were chosen and pooled in a single microfuge tube containing 0.5 ml distilled water. The samples were frozen and stored at -40^{o}C until the sample collection period was completed.

Clinical periodontal measurements

Subsequent to saliva and serum sampling, clinical periodontal recordings, including plaque index, PD, CAL, and BOP (+/-) were performed at 6 sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual locations) on each tooth present, except the third molars, using a Williams periodontal probe (Hu-Friedy, Chicago, IL, USA). CAL was assessed from the CEJ to the base of the probable pocket. BOP (deemed positive if it occurred within 15 seconds after periodontal probing) was recorded dichotomously by visual examination. All measurements were performed by two precalibrated examiners (Drs. PG and NN). Inter-examiner and intra-examiner calibration was analyzed using the Kappa-Cohen test. The initial intra-examiner kappa values were 0.96 (PD) and 0.86 (CAL) for Dr. PG and 0.93 (PD) and 0.79 (CAL) for Dr. NN. The inter-examiner values were 0.92 (PD) and 0.75 (CAL).

Plaque sample processing

Lyophilized plaque samples were weighed with an analytical microscale, then resuspended in 1X PBS to a concentration of 10 mg • ml^{-1}. Dilutions and aliquots were made and all samples were kept at -80°C before thawing for use in assays and DNA extractions. Reported patient sample numbers were ordered according to TLR4-antagonistic activities for presentation purposes and relabeled independently of the assignment number at collection.

Bacterial growth

Porphyromonas gingivalis ATCC 33277, *Fusobacterium nucleatum* ATCC 10953, and *Streptococcus gordonii* ATCC 5156 were grown in an anaerobic chamber at 37°C in the presence of 5% H_2, 5% CO_2, and 90% N_2. Bacteria were grown on blood agar (Remel) and cultured for 3-4 days before inoculating trypticase soy broth liquid cultures. *P. gingivalis* and *F. nucleatum* liquid medium was supplemented with 5 μg • ml^{-1} menadione and 1 μg • ml^{-1} hemin and adjusted to pH 7.2. For LPS extraction, *P. gingivalis* was grown in the presence of 10 μg • ml^{-1} hemin to enrich for antagonistic LPS, Pg1435LPS.

LPS extraction

Porphyromonas gingivalis and *F. nucleatum* bacterial cultures were grown to late logarithmic phase prior to harvesting. To isolate antagonistic LPS , Pg1435LPS, *P.gingivalis* were treated using a modified Darveau-Hancock LPS extraction procedure (Darveau & Hancock, 1983) followed by a phenol-water repurification (Manthey & Vogel, 1994). *F.nucleatum* agonistic LPS (FnLPS) was extracted using Tri reagent, as previously reported(Coats et al., 2009a, Yi & Hackett, 2000). Lipid A compositions were confirmed by MALDI-TOF analysis.

Dual-luciferase reporter assay

Human embryonic kidney (HEK) 293 cells were plated at a density of 4×10^4 cells per well and transfected 24 h later by calcium phosphate precipitation. Stimulations were performed, in triplicate wells, 20-24 h after transfections and were incubated for 4 h at 37°C and 5% CO_2 . For correlation studies, 1, 10 and 100 μg • ml^{-1} plaque samples were

introduced directly to serum-free DMEM containing 10% fetal bovine serum as a source of soluble CD14. For antagonisms, 100 ng • ml^{-1} FnLPS was mixed with one of following: healthy plaque samples, diseased plaque samples, Pg1435LPS or phosphate-buffered saline (PBS).

Prior to introducing plaque and/or LPS, cells were transfected with the following amount of plasmid DNA per well: pβ-actin *Renilla* Luc (0.0004 μg), pNF-κB-TA-Luc (0.02 μg), pTLR4SV1 (0.006 μg), and pMD-2 (0.0025 μg). Empty expression vector, pDisplay was used to adjust total amount of DNA per well to 0.1 μg. After 4 h stimulation, cells were rinsed with PBS and lysed with passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity of each lysate was measured using Dual Luciferase Assay Reporter System (Promega Corp., Madison, WI, USA). For correlation studies with clinical measurements, data are expressed as relative NF-kB activation, which represents the ratio of NF-kB dependent firefly luciferase activation to β-actin promoter-dependent *Renilla* luciferase activation. Antagonism data are presented as percent stimulation above FnLPS alone.

E-selectin expression assay

Human umbilical vein endothelial cells (Lonza, Rockville, MD, USA) were plated at a density of 1.4×10^4 cells per well in a gelatin-coated 96-well plate in the presence of M199 growth medium (Life Technologies, Carlsbad, CA, USA) supplemented with 4mM L-glutamine, 90 μg • ml^{-1} of heparin, 1 mM sodium pyruvate, 30 μg • ml^{-1} of endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ, USA), and 20% fetal bovine serum

(Hyclone). As with the HEK293 assays 100 ng • ml^{-1} *F. nucleatum* LPS was pre-mixed mixed with one of following: healthy plaque samples, diseased plaque samples, Pg1435LPS or PBS. Mixtures were then introduced to the cell monolayer in triplicate wells in the presence of stimulation medium supplemented with 10% human serum. After a 4 h incubation, the cells were washed with PBS, fixed in 0.5% gluteraldehyde for 10 min, then blocked in PBS containing 3% goat serum. E-selectin expression was detected by a previously described ELISA protocol(Coats et al., 2003). Antagonism data are presented as percent stimulation above FnLPS activation alone.

Genomic DNA isolation

Overnight bacterial cultures and 200 μg of plaque samples were pelleted in screw-cap microfuge tubes. The following was added to each pellet: 0.4 g of 0.1 mm zirconium/silica beads (Biospec, Bartlesville, OK, USA), 10% SDS (Sigma-Aldrich, St. Louis, MO, USA), 1X TE buffer, and TE-buffer saturated phenol, pH 8.0 (Sigma-Aldrich). Cells were disrupted in a FastPrep-24 tissue homogenizer (MP Biomedicals, Santa Ana, CA, USA) for 50 s at a speed of 5.0 M/s, then spun at top speed to separate phases. Upper aqueous phases were subject to a series of chloroform: isoamyl alcohol and phenol phase extractions. DNA was precipitated with ispropanol and 3M sodium acetate (Sigma-Aldrich) then pelleted. Pellets were washed with ethanol and allowed to air dry. All DNA were dissolved in 1x TE buffer. Concentrations of double-stranded DNA were determined by Quant-iT PicoGreen assay kit (Life Technologies, Carlsbad, CA, USA) and used to create standard curves for each target.

Quantitation of total and specific bacterial load

Absolute quantitation using qPCR was performed on a Lightcycler 480 (Roche Applied Science, Indianapolis, IN, USA). 2 µl of genomic DNA extracted from plaque or controls were added to 5 µl TaqMan Fast Advanced Master Mix (Life Technologies, Carlsbad, CA, USA), 400 nM each of forward primer and reverse primer, and 200 nM TaqMan probe (Life Technologies, Carlsbad, CA, USA). These oligonucleotide sequences are listed in Table 1. Nuclease-free water was added to bring total volume of reaction to 10 µl. Real-time PCR conditions are as follows: 50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 15 s and either 58° or 60°C for 1 min. Number of bacteria was determined with Lightcyler 480 software using Second Derivative Maximum method. Serial dilutions of genomic DNA were used for internal standard curves, which were of high quality with efficiencies of 1.8-2.0 and errors below 0.20.

Statistical analysis

To examine percent antagonism or activation of plaque samples against FnLPS in both the HEK293 and HUVEC assays, one-way ANOVA multiple comparisons with Holm-Sidak post-hoc test was used. All other tests are indicated in figure text. Analyses were performed using either GraphPad Prism v6.03 (GraphPad Software, San Diego, CA, USA) or SPSS v19.0 (IBM Corp., Armonk, NY, USA) statistical software. Differences were considered significant with $P<0.05$.

RESULTS

Characterization of healthy and diseased sites in chronic periodontitis patients

Subgingival plaque was sampled from healthy and diseased sites in 15 chronic periodontitis patients and specific clinical measurements were taken for all sites sampled. The mean measurements of PD, CAL, and PI in diseased sites were significantly greater than matched healthy sites (Table 2). Additionally, all healthy sites sampled demonstrated no bleeding on probing.

To determine the numbers of total and specific bacteria, quantitative PCR was performed on genomic DNA preps of equivalent dry weights of plaque samples. Bacterial load was significantly greater in diseased plaque with approximately 10^6 mean copy numbers, whereas healthy plaque made up more than 10-fold less bacteria (Figure 3). The compositions of each sample were also varied. *F. nucleatum* was present in both healthy and diseased samples, but in greater proportion in diseased samples (Figure 4). *Streptococcus gordonii* was detected in slightly greater amounts in healthy samples than diseased samples, whereas *P. gingivalis* is present in nearly all (11 of 15) diseased samples, reflecting the strong association between this bacterium and periodontal disease sites. Moreover, *P. gingivalis* is detected at small proportions in healthy patient samples 1H-4H and 12H.

Plaque activation of TLR2 and TLR4

To quantify the ability of matched subgingival plaque samples to activate either TLR4 or TLR2 independent of corresponding clinical measurements, we measured the NF-kB activity elicited by each sample and compared it to different clinical measurements specific to that sample. HEK cells transfected with TLR4 or 2 were treated with 1, 10 or 100 $\mu g \cdot ml^{-1}$ of sub gingival plaque sample then measured for NF-kB activity. Both TLR2 and TLR4 activities displayed a dose response, with significant decrease s at 1 $\mu g \cdot ml^{-1}$ (data not shown). The highest dose of plaque at 100 $\mu g \cdot ml^{-1}$ was chosen for further association studies with clinical measurements since activities were in the linear range of the assay which permitted direct comparison among the samples.

Plaque from diseased sites were consistently significantly more potent at both TLR4 (Figure 5a) and TLR2 (Figure 5b) whereas matched plaque from healthy sites activated both TLRs much less and with more variability at a dose of 100 $\mu g \cdot ml^{-1}$. When analyzing specifically TLR4 activation of each sample, the general trend demonstrated a significant difference between healthy sites and matched diseased sites in the same patients. These measurements established a baseline of activity to further investigate possible inhibition or antagonism of TLR4 activation and also to elucidate possible relationships with clinical measurements of disease.

Association of clinical measurements of disease to TLR2 and TLR4 activation

Since previous work has demonstrated a relationship of certain clinical measurements of supragingival plaque to its ability to stimulate TLR4 and TLR2 (Yoshioka *et al.*, 2008), we likewise determined associations, if any, of clinical measurements to the ability of our subgingival plaque samples to activate these TLRs. These correlations determined by Spearman rank are represented as r_s values (Figure 6). TLR2-specific activation had low to moderate association to BOP status (Figure 6a), PI (Figure 6b), PD (Figure 6c), and CAL (Figure 6d). There was no significant association of TLR2 activation with CAL. In contrast, TLR4-specific activation had moderate to high association to the clinical measurements (Figures 6e-h), with r_s values ranging from 0.595 (CAL) to 0.831 (PD) and *p*-values less than 0.05. These data demonstrate that both TLR2 and TLR4 activity increases in clinically diseased sites, and that the increase in TLR4 activity demonstrated a stronger correlation to disease measurements.

Plaque antagonism of TLR4 activation

Next the ability of these matched plaque samples to antagonize TLR4 activation was determined. It was hypothesized that a mixed microbial plaque sample could potentially inhibit TLR4 activation due to either the presence of antagonistic LPS (Coats et al., 2003) or lipoteichoic acid (Sugawara et al., 1999) in the sub-gingival microbial community.

In these experiments the matched plaque samples obtained from either clinically healthy or diseased sites were combined with 100 $\mu g \bullet ml^{-1}$ *F. nucleatum* LPS, in order to

determine if the plaque sample could reduce TLR4 activation in response to this TLR4 agonist. Percent stimulation above or below *F. nucleatum* LPS activation alone was determined for each of the patient plaque samples. *P. gingivalis* LPS was added as a TLR4 antagonist control. None of the sub gingival samples obtained from diseased sites displayed TLR4 antagonistic activity and in fact significantly activated TLR4 above *F. nucleatum* LPS alone (Figure 7A). In contrast, plaque samples taken at healthy sites displayed a wide range of responses from significant antagonism to significant activation (Figure 7B).

Plaque inhibition of E-selectin expression on HUVEC cells

Although using a reporter system with HEK293 cells allows for high specificity for TLR4 activation and antagonism, it may superficially represent these activities. To demonstrate TLR4 dependent antagonism in a more clinically relevant format, we measured the presence of E-selectin by endothelial cells stimulated with mixtures of the plaque and FnLPS agonist. A similar diversity of TLR4 antagonism was observed with plaque from healthy sites (Figure 8a). Moreover, three of the fifteen same samples significantly dampened the expression of E-selectin by endothelial cells after exposure for 4 hours. Also similar to the HEK-TLR4 assay, all diseased samples strongly activated E-selectin above FnLPS activation alone (Figure 8b).

DISCUSSION

This study found that sub-gingival plaque samples from diseased sites demonstrated a significant increase in both TLR2 and TLR4 activation. The increase in both TLR2 and TLR4 activation in clinically diseased sites and the significant correlations with an increase in TLR4 activity are consistent with the demonstrated increase in microbial load and the characteristic shift to gram-negative bacteria described for diseased sites (Abusleme *et al.*, 2013, Socransky et al., 1988, Marsh, 2003). As the number of bacteria increase with disease, so do the number of TLR2 and TLR4-stimulatory ligands. The slightly stronger linear association of TLR4 stimulation to clinical measurements may be partly due to the stringency of ligand recognition in lipid A from only gram-negative bacteria, whose relative abundance significantly increases in the dysbiotic community associated with periodontitis. In addition, all plaque samples from diseased sites also induced increased the TLR4 dependent expression of E-selectin in endothelial cells. This strong activation of E-selectin in disease is corroborated by a body of work demonstrating increase detection of this leukocyte extravasation molecule in inflamed tissues of chronic periodontitis patients (Rezavandi *et al.*, 2002). The clinical associations with clinical measurements described here agree with a previous study investigating supra-gingival plaque stimulation of TLR4 and TLR2 (Yoshioka et al., 2008). However, since the composition of the sub-gingival microbial community examined here is in close juxtaposition to periodontal tissue and has been found to be clinically related to the presence of periodontitis the findings of increased TLR2 and

TLR4 activation in plaque sample from diseased sites directly validates the notion that an increase in the bacterial load will result in an increased inflammatory load.

In contrast to the increased TLR4 activation observed in periodontitis affected sites, healthy sites from the same individual displayed diverse TLR4 activities including both potent agonist and antagonist activities. To our knowledge, this is the first report to describe TLR4 antagonism by *ex vivo* sub-gingival plaque clinical samples. Both HEK-TLR4 reporter and E-selectin ELISA assays demonstrated TLR4 antagonism. It is not clear if one or multiple factors contribute to the TLR4 antagonism observed in sub-gingival plaque samples obtained from clinically healthy sites. For example, within the microbial community *P. gingivalis* produces a potent TLR4 antagonistic lipid A structure that is environmentally regulated (Al-Qutub et al., 2006) and lipoteichoic acid produced by gram positive bacteria maybe present in sufficient quantities to contribute to the antagonism observed (Sugawara et al., 1999). Likewise, host components that differ in relative abundance when clinically healthy and diseased sites are compared may contribute to TLR4 antagonism. For instance, lipopolysaccharide binding protein which is expressed locally in the periodontal tissue and CD14 expression has been shown to be interrelated in periodontal tissue with significantly increased expression in clinically healthy sites (Ren et al., 2005). These key components of the TLR4 activation pathway as well as the levels of TLR4 and MD-2 may all modulate TLR4 activation.

Importantly, the stark difference in TLR4 activation observed between clinically healthy and diseased sites emphasizes the need to further define health in addition to disease

in the periodontium and the underlying mechanisms which maintain or dysregulate the homeostasis with its resident microbial community. The data suggests that TLR4 antagonism may be a normal component of healthy homeostasis, specifically antagonism may protect a healthy site from development to disease by modulating inflammatory mediator expression. For example, the sub-gingival microbial community in cooperation with locally expressed host components may regulate E-selectin whose expression is significantly increased in diseased sites (Tonetti *et al.*, 1998, Gemmell *et al.*, 1994). Additionally, E-selectin, as a key mediator of neutrophil diapedesis, may be down- regulated in clinically healthy sites to maintain the appropriate number of neutrophils to enter the gingival crevice to perform their immune-inflammatory surveillance.

In conclusion, this study characterized the functional potential of matched subgingival plaque samples from adults with chronic periodontal disease. The activation of TLR4 by subgingival plaque was associated with clinical indices. Most importantly, plaque from healthy sites was able to antagonize FnLPS activation specifically at TLR4 and dampen E-selectin expression on endothelial cells. To our knowledge, this is the first report of *ex vivo* microbial samples used to determine antagonistic potential *in vitro*. Furthermore, this type of testing may be useful for prospective clinical treatment planning. Patients who do not respond to standard therapy often benefit from antibiotic treatment resulting from microbiological testing and monitoring. Sampling subgingival plaque from all sites and testing not only microbial composition but also functionality, may be useful to predict future disease progression of a healthy site. Examination of the inflammatory activity of clinically

healthy sites as performed in this study reveals the need to more fully understand periodontal inflammatory surveillance mechanisms. Examination of diseased sites is important to study with respect to understanding mechanisms underlying the relationship between a dysbiotic periodontal community and disease. However, subtle differences in the relationship between the sub-gingival microbial community and the host such as antagonism in healthy sites may be obscured in the presence of overwhelming pro-inflammatory activities. Understanding how healthy homeostasis is maintained may lead to more effective intervention strategies for the treatment of periodontitis.

Table 1. Primer and probe sequences used for quantitative PCR analysis

Primers or Probes	Sequence (5'→3')	T_a	Product size (base pairs)	Target	Reference
Universal					
Forward	CGCTAGTAATCGTGGATCAGAATG			16s	(Yoshida *et al.*,
Reverse	TGTGACGGGCGGTGTGTA	58°C	69	rDNA	2003)
TaqMan Probe	6FAM-CACGGTGAATACGTTCCCGGGC-TAMRA				
P. gingivalis					
Forward	TGCAACTTGCCTTACAGAGGG				(Gaetti-Jardim *et*
Reverse	ACTCGTATCGCCCGTTATTC	60°C	126	*waaA*	*al.*, 2009)
TaqMan Probe	6FAM-AGCTGTAAGATAGGCATGCGTCCCATTAGCTA-TAMRA				
S. gordonii					
Forward	CGGATGATGCTAATCAAGTGACC				(Alvarez *et al.*,
Reverse	GTTAGCTGTTGGATTGGTTGCC	60°C	177	*gtfG*	2013)
TaqMan Probe	6FAM-AGAACAGTCCGCTGTTCAGAGCAA-TAMRA				
F. nucleatum					
Forward	TGCAGCAAGTTTAGTAGGTG				(Alvarez et al.,
Reverse	CATTGTAAACTTGTTCATTTTGT	60°C	146	*fadA*	2013)
TaqMan Probe	6FAM-AGCACTAGATGCTGAATACCAA-TAMRA				

Table 2. Summary of clinical characteristics of matched healthy and diseased sites from subjects. Values represent the mean for all the measured sites in the subjects, but individual means were used for paired t-tests. *P<0.05, **P<0.01

	Healthy	Diseased
	N=15	N=15
Probing Depth (mean ± SD)	2.2 ± 0.4	5.9 ± 1.8**
Clinical Attachment Level (mean ± SD)	3.7 ± 0.9	7.5 ± 1.2**
Plaque Index (mean ± SD)	1.4 ± 0.5	2.8 ± 0.4*
Bleeding on Probing status	-	+

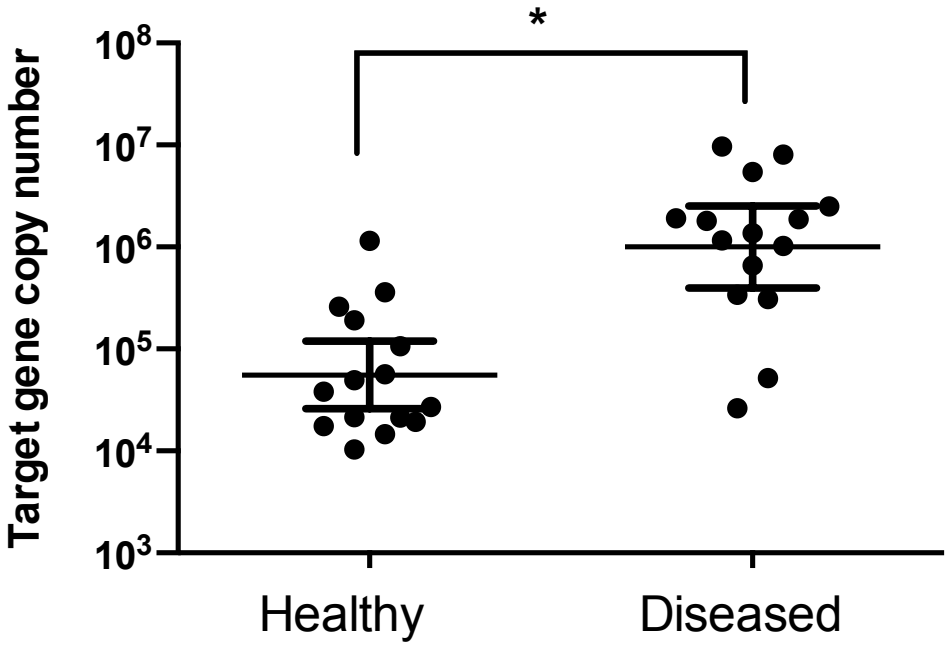


Figure 3. Bacterial load of matched healthy and diseased plaque samples. The copy number of 16s rRNA genes represent the total bacterial load for each sample. Differences were tested using Wilcoxon matched-pairs signed-rank test with significance of *P<0.05.

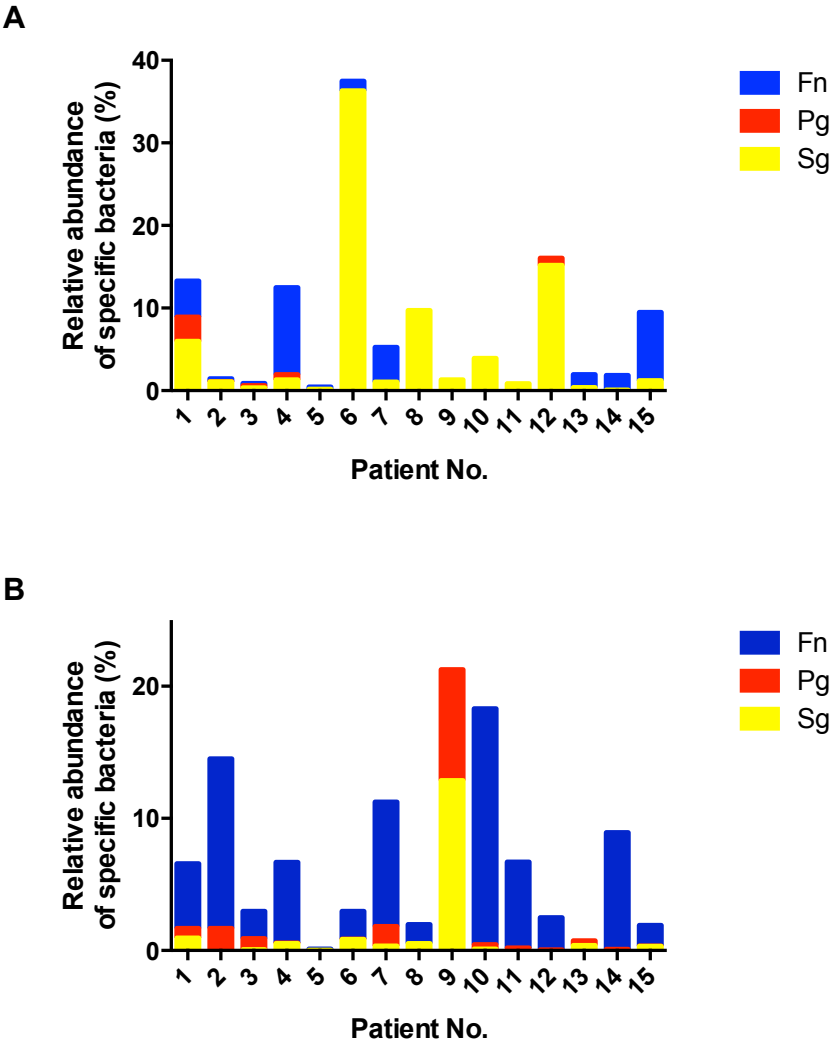


Figure 4. Relative abundance of *F. nucleatum* (Fn), *P. gingivalis* (Pg) and *S. gordonii* (Sg) in healthy (A) and diseased (B) patient samples.

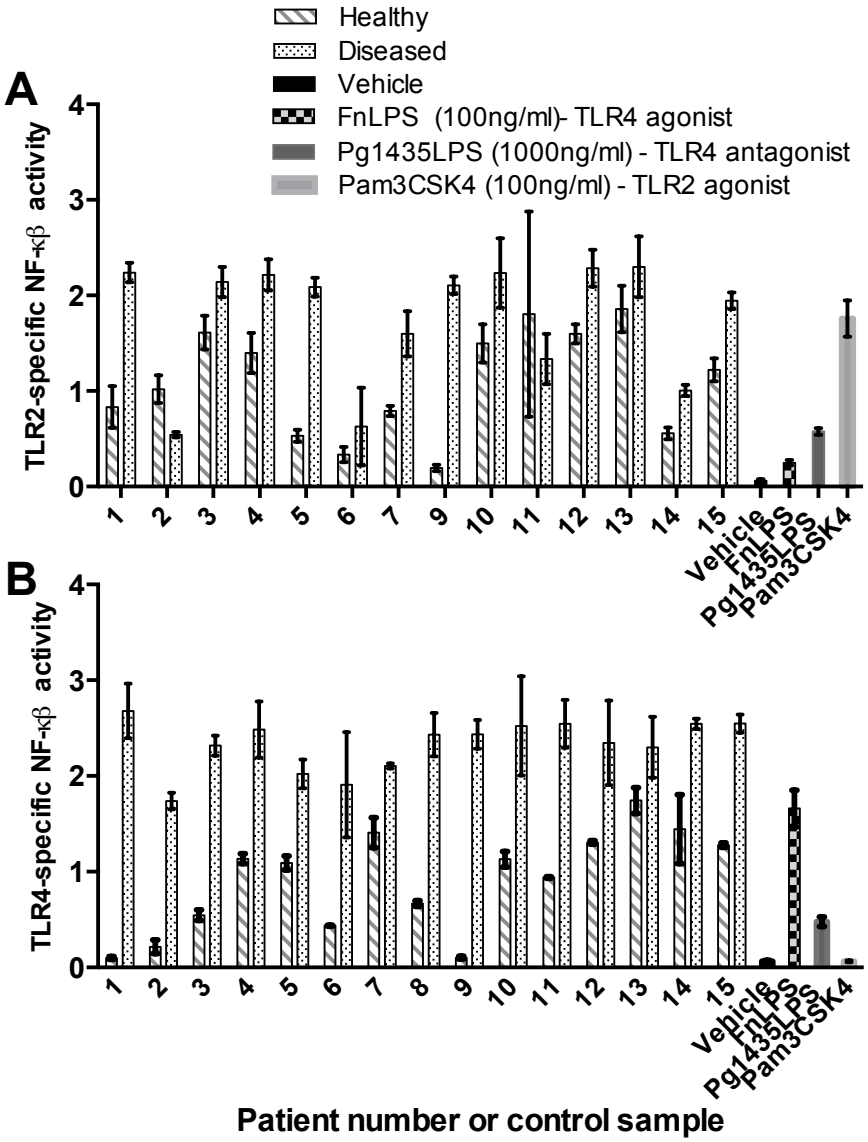


Figure 5. TLR4 (A) and TLR2 (B) activation by subgingival plaque from matched healthy or diseased sites in chronic periodontitis patients. 100 μg • ml^{-1} of either matched healthy (A) or diseased (B) plaque were tested in triplicate wells in three independent assays (N=3). Relative TLR4 activation was determined by the ratio of NF-κB-dependent firefly luciferase activity to β-actin promoter-dependent Renilla luciferase activity. Error bars indicate 95% confidence intervals.

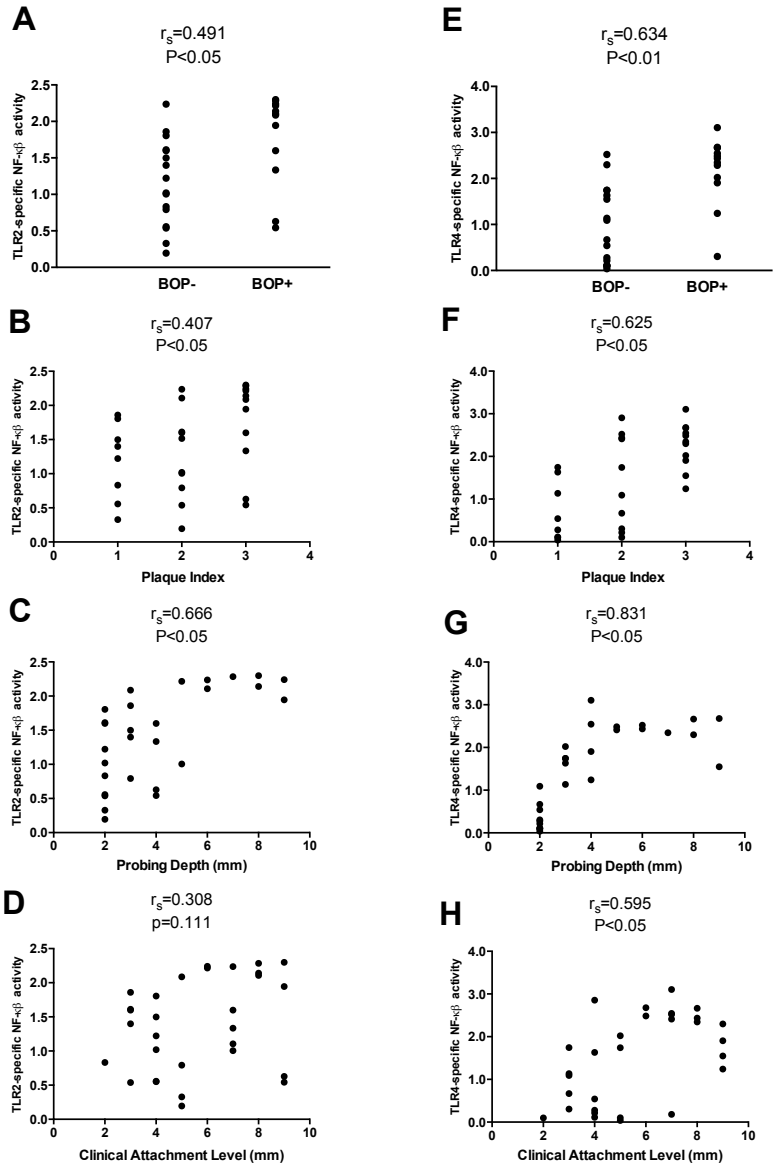


Figure 6. Relationships between TLR2 or TLR4-specific activation and clinical measurements of chronic periodontitis. TLR2 (A-D) and TLR4 (E-H) activation is represented by relative NF-kB activation and associations were determined with corresponding clinical measurements: BOP status (A, E), plaque index (B, F), probing depth (C, G), and clinical attachment levels (D, H). Correlation was determined by Spearman rank (r_s) with significance of $*P<0.05$.

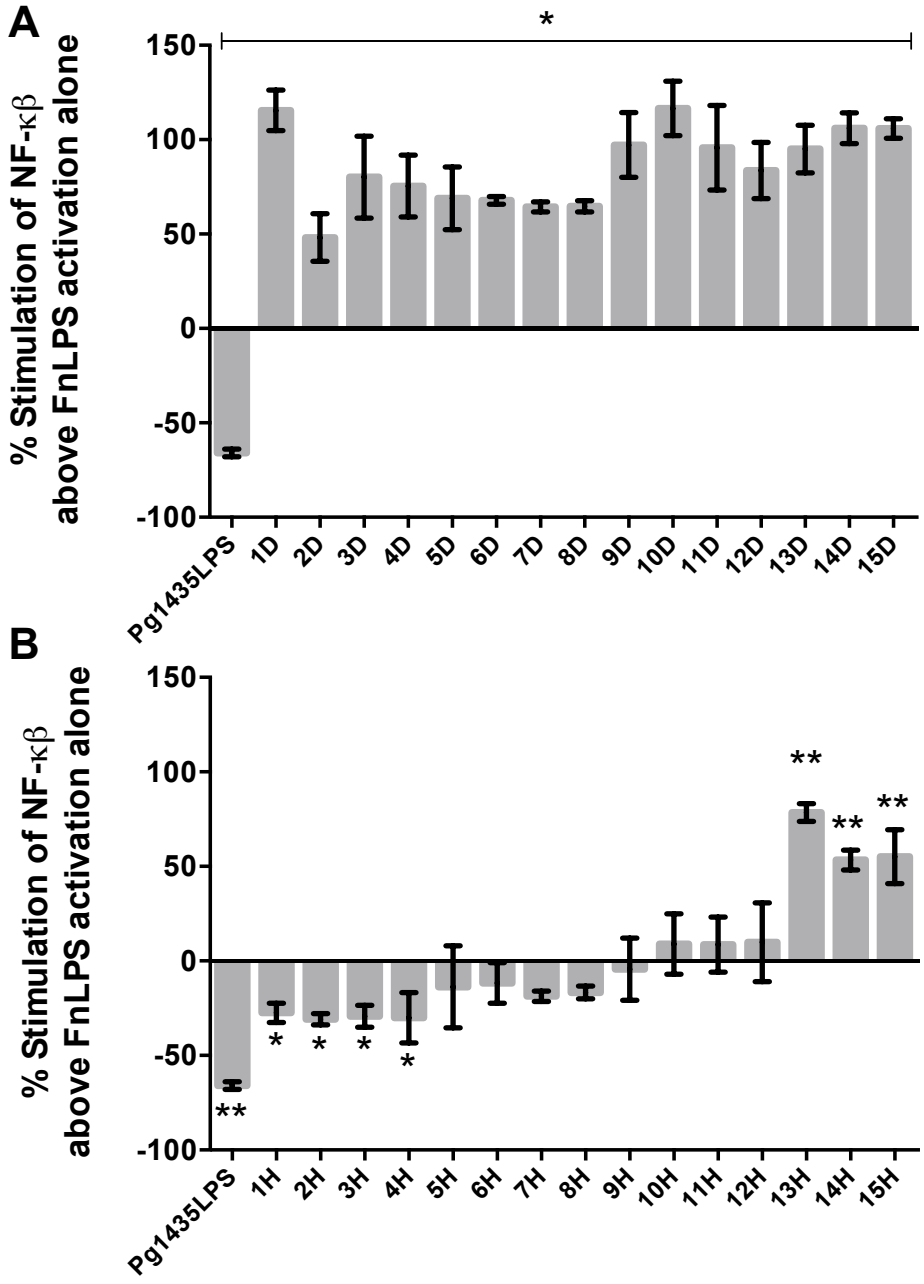


Figure 7. TLR4 antagonistic or stimulatory potential of subgingival plaque. Percent stimulation of NF-kB above F. nucleatum LPS (FnLPS) activation alone was calculated using the formula $[((y-x))/x]\times 100$, where x is NF-kB activity by TLR4 stimulation with 100 ng/ml FnLPS alone and y is FnLPS combined with either 100 μg • ml-1 plaque or 1 μg • ml-1 l Pg1435LPS control. Matched healthy (A) and diseased (B) plaque were tested in triplicate and the data is representative of three independent experiments. *P<0.05; **P<0.01

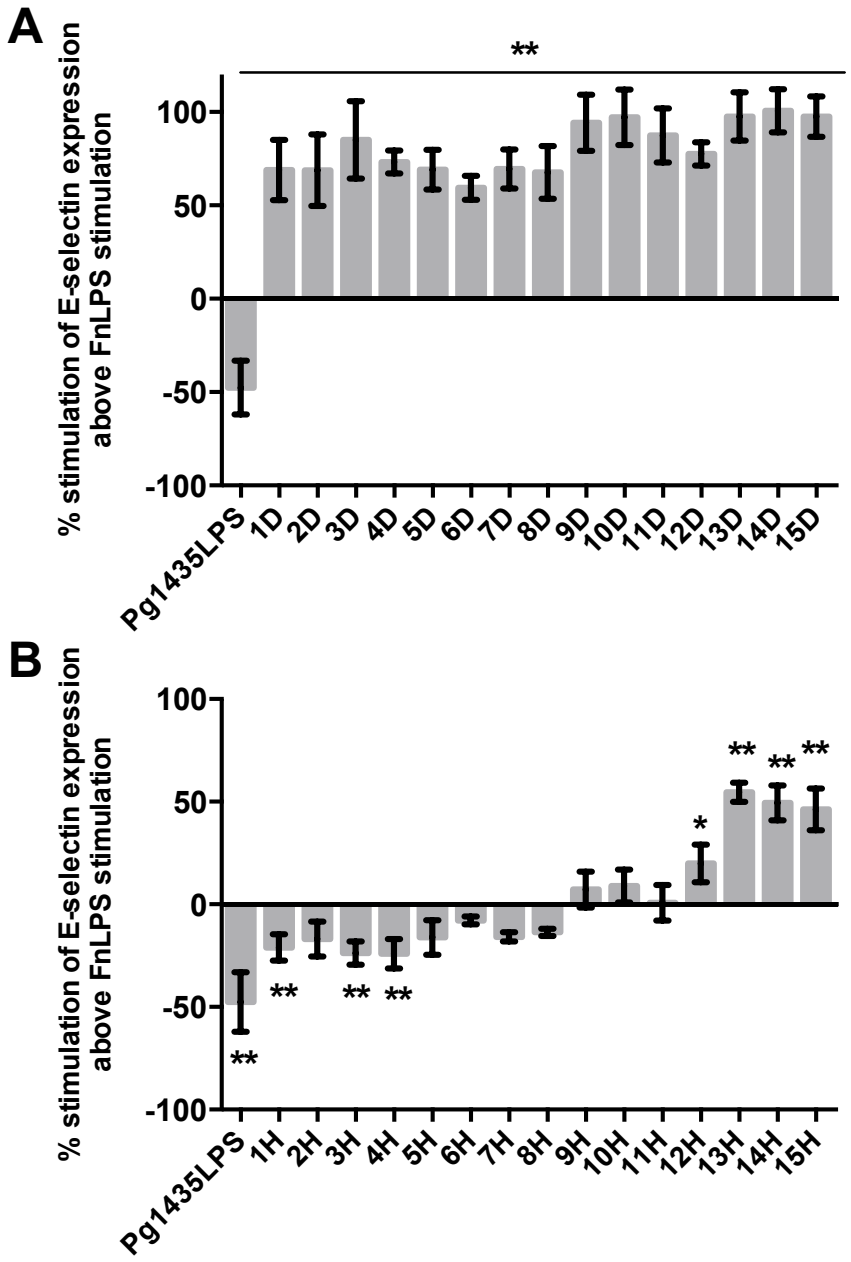


Figure 8. Antagonism and stimulation of E-selectin expression of endothelial cells by subgingival plaque. Percent stimulation of E-selectin expression above FnLPS activation alone was calculated as before (Figure 7). Matched healthy (A) and diseased (B) plaque were tested in triplicate and data is representative of three independent experiments. *P<0.05; **P<0.01

CHAPTER III: *F. NUCLEATUM* ALTERS ITS LIPID A IN RESPONSE TO THE MICROBIAL COMMUNITY

INTRODUCTION

Fusobacterium nucleatum maintains a unique role in the periodontal microbial community and is prevalent during health and disease, encouraging plaque development by co-aggregating with both commensal and pathogenic bacterial species (Bradshaw et al., 1998). A recent study has evaluated lipid A compositional changes of *P. gingivalis* when co-cultured with *F. nucleatum*. Curiously, this work did not characterize the lipid A of *F. nucleatum* (Lee & Baek, 2013). We postulated that *F. nucleatum* could likewise alter its lipid A composition in the presence of *P. gingivalis* and that these changes could potentially impact TLR4 activation. Additionally, we formulated an improved MALDI-TOF matrix suitable for detection of highly heterogeneous lipid A mixtures. Together, our results demonstrate how two disease-associated co-aggregating oral bacteria, *F. nucleatum and P. gingivalis* synergistically potentiate a pro-inflammatory response through TLR4-specific activation by lipid A modification and also how we can better monitor these highly diverse structural changes through improved mass spectrometry preparation.

MATERIALS AND METHODS

Bacterial strains and co-culture planktonic growth

P. gingivalis 381 wild-type, originally from Caroline Genco lab, and lipid A phosphatase mutants were created and provided by Stephen Coats. *F. nucleatum* ATCC

25586, *P. gingivalis* ATCC 33277, and *S. gordonii* ATCC 51656 (American Type Culture Collection, Manassas, VA, USA) , in addition to *P. gingivalis* 381, were grown in an anaerobic chamber at 37°C in the presence of 5% H_2, 5% CO_2, and 90% N_2. Bacteria were grown on blood agar (Remel, Lenexa, KS, USA) and cultured for 3-4 days before inoculating trypticase soy broth liquid cultures, supplemented with 5 μg • ml^{-1} menadione and 1 μg • ml^{-1} hemin and adjusted to pH 7.2, otherwise known as TYHK medium

For co-culture studies, overnight mono-culture starters of each bacterial strain were grown and the optical density at wavelength 600 (OD600) was measured and concentration of each was estimated, assuming an OD600 of 1 equaled approximately $1x10^9$ bacterial cells/ mL. A final 200 mL of TYHK medium was inoculated with following ratios of *F. nucleatum* to *P. gingivalis* or *S. gordonii* cell concentrations: approximately 3:1, 1:1, and 1:3. Equivalent concentrations of *F. nucleatum* were inoculated into TYHK media with and without supplementation of 1% propionic acid and simultaneously cultured and used in structure and function studies.

LPS purification and lipid A isolation

LPS was isolated using a modified version of the TRI-reagent protocol for LPS isolation, as described previously (Yi & Hackett, 2000, Al-Qutub et al., 2006). 200 mL of late-logarithmic bacterial culture was centrifuged at 6, 500 rpm. And the bacterial pellet was rinsed once with endotoxin-free water. After this, the pellet was re-suspended in 1 mL of TRI reagent (Molecular Research Center, Inc., Columbus, OH, USA), a commercial

preparation of guanidine thiocyanate and phenol. After thorough homogenization by vortexing, chloroform was added at one-fifth of the volume and further homogenized. The mixture was centrifuged for 10 minutes at 12, 000 rpm and the top, aqueous layer containing LPS was retained. Endotoxin-free water was used to re-extract remaining LPS from the remaining intermediate and organic phases once more and the pooled aqueous phases were lyophilized, yielding "crude LPS". Crude LPS was then washed once with cold 0.375M MgCl2 in 95% ethanol, centrifuged at 4 °C at 5, 000 rpm for 5 minutes and washed three additional times with 95% ethanol, followed by a final was with cold 100% ethanol. After evaporation of residual ethanol, LPS was subjected to a modified Folch extraction to remove contaminating phospholipids (FOLCH *et al.*, 1957). Briefly, crude LPS was washed in chloroform/methanol (2:1, v/v) and then dried, prior to re-suspension in water and lyophilization, yielding "purified LPS".

Hydrolysis of purified LPS to isolate lipid A was accomplished using a mild acid/detergent treatment (Caroff *et al.*, 1988)in the presence of heat (Caroff et al., 1988). Specifically, 1-3 mg of dried LPS was resuspended in 10 mM sodium acetate [pH 4.5] containing 1% sodium dodecyl sulfate (w/v). This mixture was incubated on heat block at 100C for 1 hour followed by lyophilization overnight. The resulting lipid A pellets were washed once in cold 95% ethanol containing 0.02 N HCl, three times in 95% ethanol. The recovered lipid A (1-2 mg) was then followed by a final Bligh-Dyer extraction (Bligh & Dyer, 1959) in a solution of chloroform/methanol/water (4:1:1, v/v/v). The solution was mixed vigorously and centrifuged at 5, 000 rpm for 5 minutes at room temperature. The top

aqueous layer was discarded and the bottom organic layer was considered to contain purified lipid A. The bottom, organic phase containing the lipid A was dried under a stream of nitrogen followed by suspension in water and lyophilized to concentrate the purified lipid A.

Development of novel MALDI-TOF matrix for enhanced lipid A detection

We first sought a method to enhance sensitivity of our heterogeneous lipid A mixtures by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) through modification of the matrix material. Heterogeneous mixtures extracted from *P. gingivalis* lipid A phosphatase mutant cultures were analyzed using mixtures of Norharmane matrix (Figure 9C) with and without the addition of prepared NH_4-EDTA additive. Similarly, lipid A from *F. nucleatum* was analyzed for improvements in peak intensity and detection. To prepare the ammonium-EDTA salt, 700 mg of EDTA acid (Sigma-Aldrich, St. Louis, MO, USA) was solubilized in a 30% ammonium hydroxide solution. The mixture was frozen and lyophilized. To prepare the matrix, the NH_4EDTA salt was added to Norharmane (Sigma-Aldrich, St. Louis, MO, USA) and solubilized in chloroform/methanol (1:1, v/v) and large precipitates allowed to settle before using the solubilized matrix for co-crystallization with the lipid A on the MALDI target plate.

MALDI-TOF mass spectrometry of lipid A

Lipid A was analyzed by MALDI-TOF in the negative ion mode on an Autoflex II (Bruker Daltonics, Billerica, MA, USA). Lipid A (0.2 mg) was dissolved in 10 uL of

chloroform and 1.0 uL was spotted directly on to the MALDI target plate and the solvent was evaporated by ambient air. Next, 1.0 uL of the Norharmane +NH_4EDTA matrix was overlaid directly on top of the sample spot and again the solvent was allowed to evaporate, leaving behind the co-crystallized matrix/ sample spot. The MALDI target plate was then placed in the ionization source to be analyzed by MALDI-TOF MS in negative-ion mode. MS data was acquired in reflectron mode with a Nd:YAG laser with 50Hz repetition rate and up to 3000 shots were accumulated for each spectrum. Instrument calibration and all other tuning parameters were optimized using ESI Tuning Mix (Agilent Technologies, Palo Alto, CA, USA). Data was acquired and processed using flexAnalysis software (Bruker Daltonics, Billerica, MA, USA)

Dual-luciferase reporter assay

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA, USA) were plated at a density of 4×10^4 cells per well and transfected 24 h later by calcium phosphate precipitation. Stimulations were performed, in triplicate wells, 20-24 h after transfections and were incubated for 4 h at 37°C and 5% CO_2. LPS was mixed endotoxin-free water and serially diluted prior to stimulation of HEK293 cells. For whole bacteria, inoculated as described above, OD600 was measured once again and 1 mL was briefly centrifuged to form a pellet. The pellet was re-suspended in 1 mL endotoxin-free water and serial dilutions prepared in assay stimulation media to achieve final numbers of approximately 10^5, 10^6, and 10^7 bacterial cells.

Prior to introducing LPS or whole bacteria, HEK293 cells were transfected with the following amount of plasmid DNA per well: pβ-actin Renilla Luc (0.0004 μg), pNF-κB-firefly Luc (0.02 μg), pTLR4SV1 (0.006 μg), and pMD-2 (0.0025 μg). Empty expression vector, pDisplay was used to adjust total amount of DNA per well to 0.1 μg. After 4 h stimulation, cells were rinsed with PBS and lysed with passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity of each lysate was measured using Dual Luciferase Assay Reporter System (Promega Corp., Madison, WI, USA). Values are reported as the fold increase of relative luciferase units (firefly luciferase/ Renilla luciferase) for the LPS-stimulated samples compared to the non-stimulated control response, whose ratio was set at 1.

RESULTS

NH_4EDTA matrix additive enhances lipid A MALDI-TOF peak detection

To initially examine the effect on known highly heterogeneous lipid A mixtures, we extracted lipid A from *P. gingivalis* 381 wild-type and mutants deficient in the lipid A modification genes. The lipid A composition of these are similar to those of another *P. gingivalis* strain, ATCC 33277, published previously using a CMBT matrix (Coats et al., 2011). Nominal masses of lipid A peaks are reported and labeled (Figure 10). Co-crystallized with norharmane matrix only, wild-type yielded major peak clusters at m/z 1368, 1448, and 1688 (non-phosphorylated, mono-phosphorylated/tetra-acylated, and mono-phosphorylated/penta-acylated, respectively) (Figure 10A). With addition of co-matrix, we

were able to detect an additional major cluster at *m/z* 1768 (di-phoshorylated lipid A), without loss of detection for the other lipid species (Figure 10D). Most remarkably, we not only detected presence of *m/z* 1768 for both lipid A phosphatase mutants, where there was no detection without the co-matrix additive, we could also detect a new, distinct peak cluster which differs from the *m/z* 1768 peak by one phosphoethanolamine (PEtN) residue (10E).

Additionally, we applied this matrix formulation to lipid A of our bacterium of interest, *F. nucleatum*. Again, equivalent amounts of lipid A was spotted on to the MALDI target plate and different matrix formulations tested. Although no significant compositional changes were detected, overall signal intensity was improved by ~8 –fold in *F. nucleatum* grown in mono-culture and ~30 percent with lipid A from *F. nucleatum* grown in presence of propionic acid (Figure 11).

F. nucleatum *alters its lipid A in response to P. gingivalis and major by-product, propionate*

Since *F. nucleatum* is highly associated with *P. gingivalis* in a dysbiotic microbial community, we co-cultured these two bacterium together and also supplemented *F. nucleatum* growth media with 1% propionic acid, a major fermentation by-product produced by *P. gingivalis (Stehle et al., 2001). F. nucleatum* in mono-culture yields primarily two lipid A peaks: *m/z* 1882 and 1910 (Figure 12d). In the presence of *P. gingivalis, F. nucleatum* produces a defined peak cluster around *m/z* 1882, with peaks differing in mass by 14 amu. Similarly, in the presence of propionic acid, *F. nucleatum* likewise increases its lipid A heterogeneity, displaying a unique peak cluster (Figures 12b,c). However, *P. gingivalis* lipid A is not

significantly altered in co-culture with *F. nucleatum*. Furthermore, the formation of lipid A clusters was observed in *F. nucleatum subsp. nucleatum* ATCC 23726, demonstrating this phenotype is conserved among different strains of *F. nucleatum* and not isolated to the ATCC 25586 strain (Figure 16).

F. nucleatum *does not alter its lipid A in response to S. gordonii nor its major byproduct, lactic acid*

To demonstrate how a metabolic relationship associated with a healthy microbiota affects *F. nucleatum* lipid A, we co-cultured *F. nucleatum* with *S. gordonii*, which it strongly co-aggregates with in dental plaque (Jang *et al.*, 2013). Unlike the *F. nucleatum-P.gingivalis* co-culture, this co-culture demonstrated no change to *F. nucleatum* lipid A composition. Additionally, *F. nucleatum* grown in the presence of TYHK medium supplemented with 1% lactic acid, a major fermentation by-product of *S. gordonii* (Egland et al., 2004), demonstrated no alterations to its lipid A content (Figure 14).

Increase of lipid A heterogeneity increases potency of TLR4 activation

To compliment structural characterization of *F. nucleatum* lipid A, we evaluated alterations to functional ability to activate TLR4. In the presence of *P. gingivalis* or propionic acid, *F. nucleatum* LPS activated TLR4 up to 10-fold greater than without either of these additions (Figure 13a). Whole bacterial stimulation reflects this activation pattern with LPS (Figure 13b). The data presented in Figure 13 represents *F. nucleatum* grown in planktonic culture with *P. gingivalis* at an initial inoculation concentration ratio of 3:1.

Conversely, neither LPS nor whole bacteria *F. nucleatum* co-cultured with *S. gordonii* or lactic acid activated significantly above *F. nucleatum* mono-culture alone (Figure 15). The only exception, however, is whole bacterial stimulation with *F. nucleatum-S. gordonii* co-culture at 10^6 bacteria. This reflects a reduction of number of *F. nucleatum* bacteria overall as opposed to alterations to LPS, since stimulation doses are based OD600 measurements. Similarly, *F. nucleatum-P.gingivalis* co-culture also reflects overall reduction of *F. nucleatum* compared to mono-culture, but in this case, the relationship results in *increased* activation of TLR4 and displays the strength of *F. nucleatum* lipid A modifications on increased TLR4 activation despite reduced bacterial numbers.

DISCUSSION

Improvements in heterogenerous lipid A detection

This work demonstrates the significant advantage of matrix additives, namely NH_4EDTA, for enhanced lipid A detection by MALDI-TOF MS. This additive can be supplemented to various matrices to increase overall signal intensity and provide tolerance to salts and detergents either natively present in a sample or added due to the lipid extraction procedure. Previous work has identified enhancement of lipid detection by MALDI-TOF with by EDTA ammonium salt additive to CMBT matrix (Figure 9b) (Zhou *et al.*, 2010). However, simultaneous visualization of non-phosphorylated lipid A species alongside phosphorylated variants is inconsistent and often requires switching from negative to positive ion mode (Coats et al., 2009b). Instead, recent use of Norharmane matrix, which is

also soluble in a solvent compatible with lipid A, provides overall more sensitive detection of heterogeneous lipid A mixtures (Curtis et al., 2011).

The sensitive detection of phosphoethanolamine (PEtN) modification in our studies is particularly remarkable. Natural PEtN addition is present in the lipid A of many serotypes of enterohaemorrhagic *E. coli,* including 0157:H7*,* and absent from the lipid A of *E. coli* K-12 (Kim *et al.*, 2006). Additionally, PEtN-modified lipid A species may confer resistance to killing by cationic antimicrobial compounds, likely by masking negatively-charged phosphoryl groups, likely at the C1 position of the reducing sugar, allowing a bacterium to grow slightly under the radar of innate immune responses (Kim et al., 2006). Enhanced detection methods, such as the modification to a MALDI-TOF matrix formulation shown in this work, can improve signal intensity and peak detection of highly heterogenous lipid A mixtures, allowing for more robust and rapid identification of both single pathogenic species and incredibly diverse polymicrobial infections.

Fn is altered in both lipid A composition and TLR4 activation when associated with Pg.

F. nucleatum and particularly *P. gingivalis* are individually associated with periodontal disease. There is, however, an even stronger correlation between the presence of these two species together in the plaque community and an increase in clinical measurements indicative of disease (Socransky et al., 1988, Kolenbrander et al., 2006). Additionally, there is a body of work demonstrating the increased production of short-chain fatty acids, particularly propionic acid, in gingival crevicular fluid of chronic periodontitis patients (Niederman *et al.*,

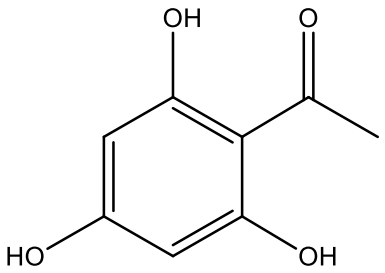
1997, Singer & Buckner, 1981, Tonetti *et al.*, 1987, Yu *et al.*, 2014). Like other anaerobes, oral bacteria thrive in anaerobic conditions and produce these short-chain carboxylic acids as by-products of fermentation. Although the previously mentioned studies demonstrate the role of propionic acid and other short-chain fatty acids on different host responses, what is not well understood is how these specific metabolic by-products affect other neighboring species in the polymicrobial community. Our results demonstrate that *F. nucleatum* grown in the presence of propionic acid and *P. gingivalis* can activate TLR4 more potently than it can alone (Figure 13), whereas growth in the presence of *S. gordonii*, a relationship more associated with a healthy microbiota, does not significantly change *F. nucleatum* activation of TLR4.

Although this is the first report of *F. nucleatum* lipid A modification, it certainly is not the first report of lipid A MS peak clustering. The peaks within each cluster differ by 12 amu, equivalent to one methylene unit added to or removed from the end of a fatty-acyl chain. Although *E. coli* LpxA, the enzyme responsible for acylating the lipid A precursor molecule, UDP-GlcNac, is highly selective for C14 fatty acyl chains, in the presence of propionic acid in the growth medium, the bacteria will produce lipid A with not only C14 fatty acyl chains, but also C13 and C15. Furthermore, *P. gingivalis* constitutively produces lipid A peaks which are clustered. In the presence of propionic acid in the medium, this clustering is more pronounced (Bainbridge *et al.*, 2008). Also, amino acid sequence alignment of the active sites of LpxA from species with different lipid A fatty acid lengths display significant residue differences, altering each species "hydrocarbon ruler" within the pocket of its respective LpxA (Wyckoff *et al.*, 1998)(Wyckoff *et al.*, 1998)

Lastly, lipid A from *Bacteroides* and *Prevotella* species likewise consistent production of lipid A "clusters", rather than a primarily singular, monoisotopic peak (Chilton *et al.*, 2013, Berezow et al., 2009).

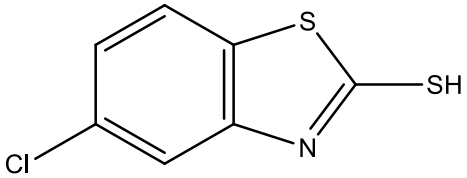
Curiously, we did not identify significant lipid A alterations to *P. gingivalis* lipid A in the presence of *F. nucleatum*. This is in contrast to a previous report, which identified greater amounts of weak agonist *P. gingivalis* lipid A (m/z 1690) relative to TLR4-silent (m/z 1250) and TLR4-antagonistic lipid A (m/z 1450) in co-culture with *F. nucleatum* (Lee & Baek, 2013). Upon careful examination of the mass spectra and x-axis scale, the previous published study incorrectly labeled a strong TLR4 agonist lipid A (m/z 1768) as the weak agonist lipid A (m/z 1690). An increase in production of a strong TLR4 stimulatory lipid A more strongly supports both their bioactivity findings, with increased expression of IL-6, IL-8, and IL1-β by *P. gingivalis* in co-culture, and our observation that this particular polymicrobial relationship potentiates a synergistic pro-inflammatory response by the innate immune system (Figure 17). In conclusion, our study underscores the need to identify and characterize polymicrobial metabolic interactions and how these relationships affect health and disease states in the host.

A



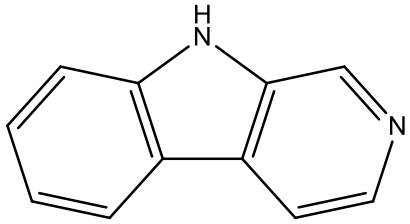
Chemical Formula: $C_8H_8O_4$
Exact Mass: 168.04

B



Chemical Formula: $C_7H_4ClNS_2$
Exact Mass: 200.95

C



Chemical Formula: $C_{11}H_8N_2$
Exact Mass: 168.07

Figure 9. Common matrices used for detection of lipid A by MALDI-TOF MS include 2',4',6'-trihydroxyacetophenone monohydrate or THAP (A), 5-chloro-2-benzothiazolethiol or CMBT (B), and beta-Carboline or Norharmane (C).

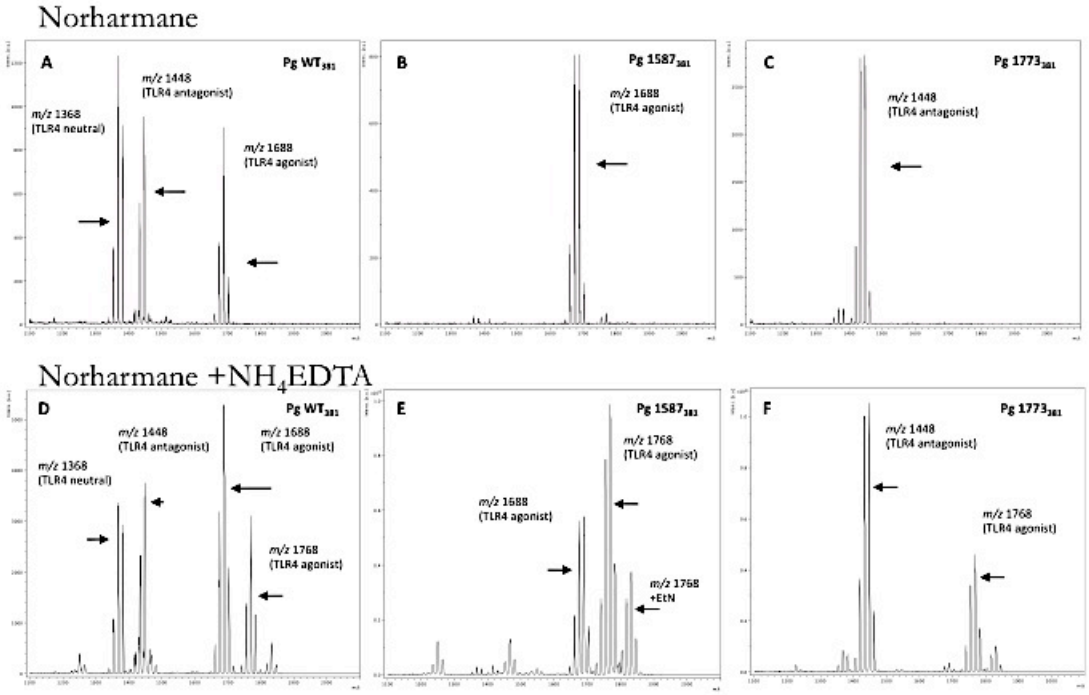


Figure 10. The effect of addition of NH_4EDTA to Norharmane matrix on MALDI-TOF detection of *P. gingivalis* 381 lipid A phosphatase mutants: Wild-type (A, D), Δ1587 (B, E), and Δ1773 (C, F). Addition of 20mM NH_4EDTA not only increases overall signal intensity (up to 4-fold) but also reveals novel lipid A peaks not detected by Norharmane matrix alone.

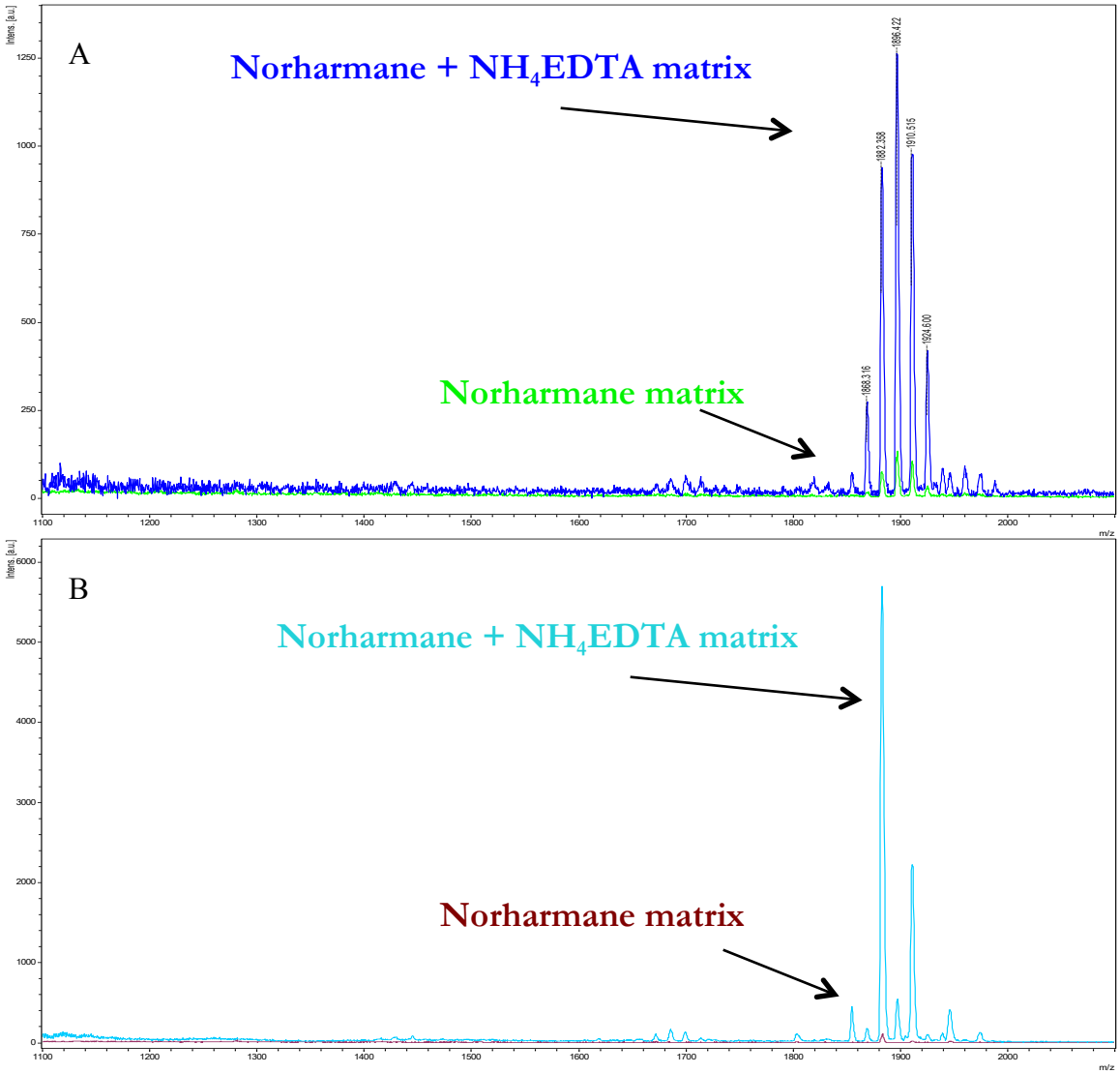


Figure 11. NH_4EDTA matrix additive significantly enhances detection of one equivalent amount of lipid A extracted from *F. nucleatum* ATCC 25586 either grown with (A) and without (B) propionic acid. Peak intensity is improved up to 30-fold with addition of matrix additive to Norharmane.

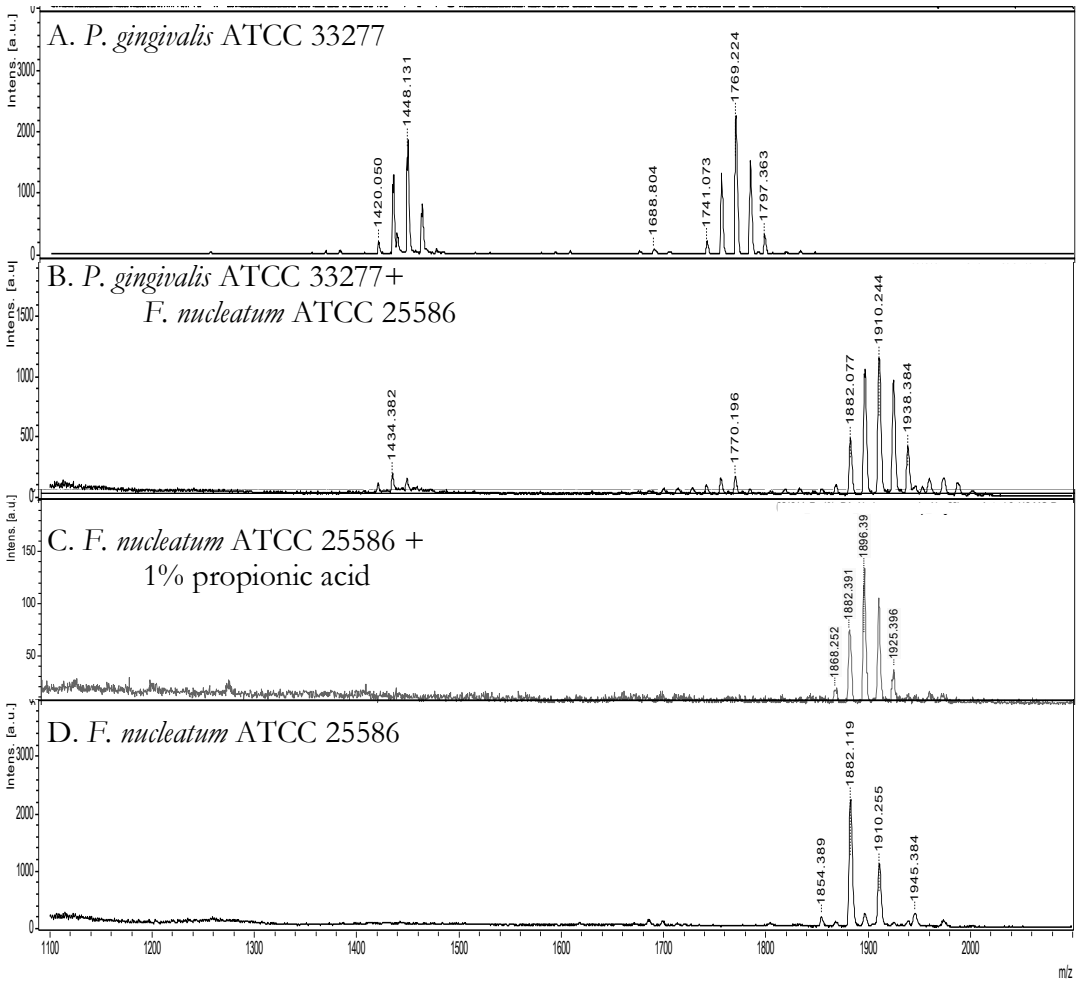


Figure 12. Negative ion mode MALDI-TOF mass spectra of the lipid A of *P. gingivalis* ATCC 33277(A) and *F. nucleatum* ATCC 25586 (D) grown in monoculture and *F. nucleatum* grown in the presence of *P. gingivalis* (B) or 1% propionic acid supplemented to TYHK growth medium (C). In the presence of the latter two additions, *F. nucleatum* significantly alters its lipid A, forming lipid A "clusters".

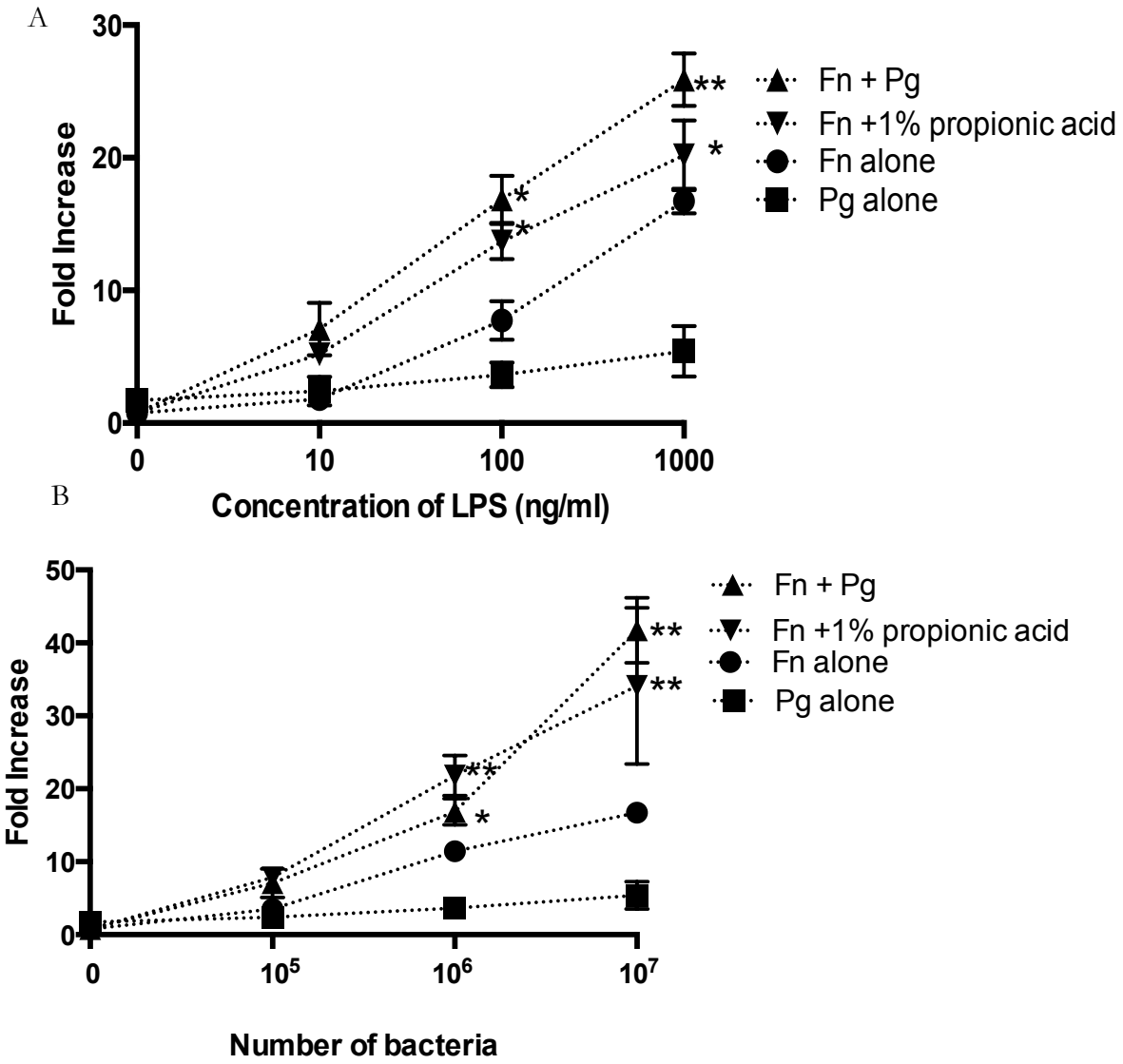


Figure 13. NF-kB activation of TLR4-transfected HEK293 cells following LPS stimulation. TLR4-trasnfected HEK293 cells were stimulated with LPS (A) or whole bacteria (B) of the following bacteria and growth conditions: *P. gingivalis* ATCC 33277(■) and *F. nucleatum* ATCC 25586 grown in monoculture (●)and *F. nucleatum* grown in the presence of *P. gingivalis* (▲) or 1% propionic acid supplemented to TYHK growth medium (▼). Values are reported as the fold increase of relative luciferase units (firefly luciferase/ Renilla luciferase) for the LPS-stimulated samples compared to the non-stimulated control response, whose ratio was set at 1. Results reported are means and standard deviations of triplicate wells and representative of at least two independent experiments.*P<0.05, **P<0.01

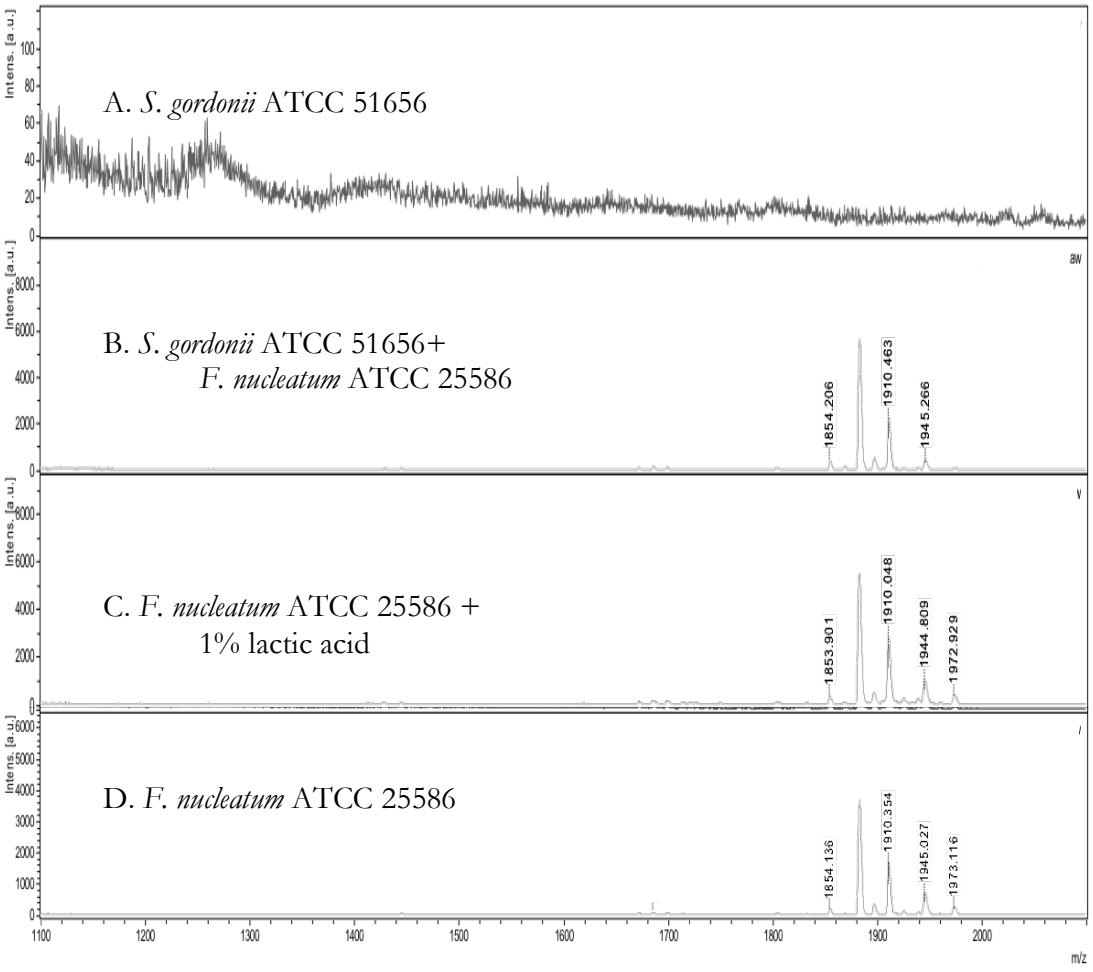
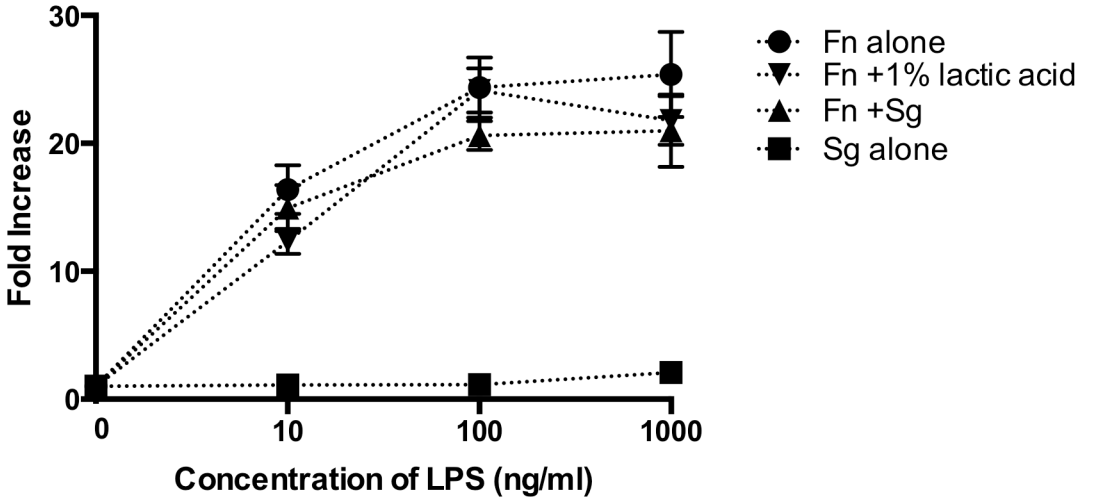


Figure 14. Negative-ion MALDI-TOF mass spectra of lipid A extracted from *S. gordonii* ATCC 51656 in mono-culture (A) or *F. nucleatum* ATCC 25586 grown in the presence of *S. gordonii* (B), lactic acid (C), or in mono-culture (D).

A



B

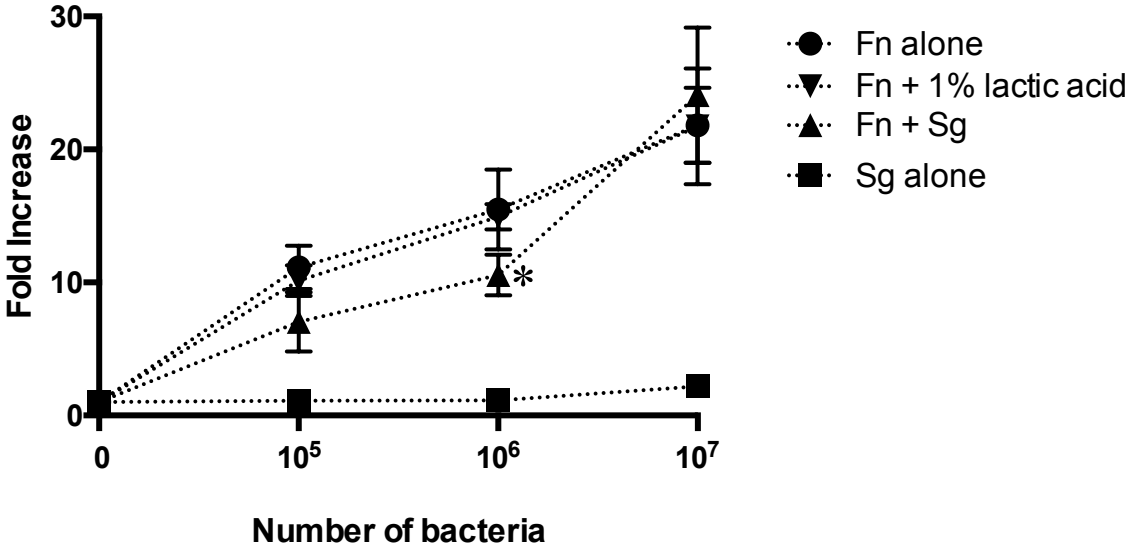


Figure 15. NF-kB activation of TLR4-transfected HEK293 cells following LPS stimulation. TLR4-trasnfected HEK293 cells were stimulated with LPS (A) or whole bacteria (B) of the following bacteria and growth conditions: *S. gordonii* ATCC 51656 (■) and *F. nucleatum* ATCC 25586 grown in monoculture (●) and *F. nucleatum* grown in the presence of *S. gordonii* (▲) or 1% lactic acid supplemented to TYHK growth medium (▼). Values are reported as the fold increase of relative luciferase units (firefly luciferase/ Renilla luciferase) for the LPS-stimulated samples compared to the non-stimulated control response, whose ratio was set at 1. Results reported are means and standard deviations of triplicate wells and representative of at least two independent experiments.*$P<0.05$

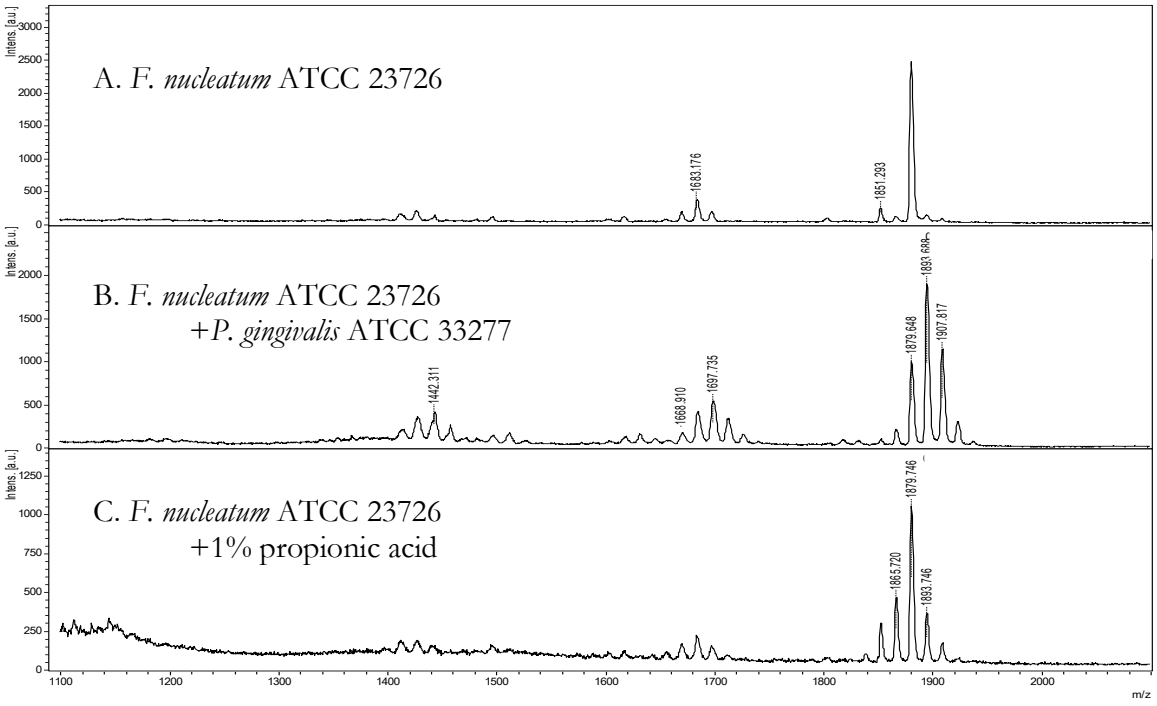


Figure 16. Negative ion MALDI-TOF mass spectra of *Fusobacterium nucleatum* ss. nucleatum ATCC 23726 lipid A in mono-culture (A) and in response to co-culture with P. gingivalis ATCC 33277 (B) and in the presence of excess propionic acid (C). Similar to ATCC 25586, this strain increases lipid A heterogeneity.

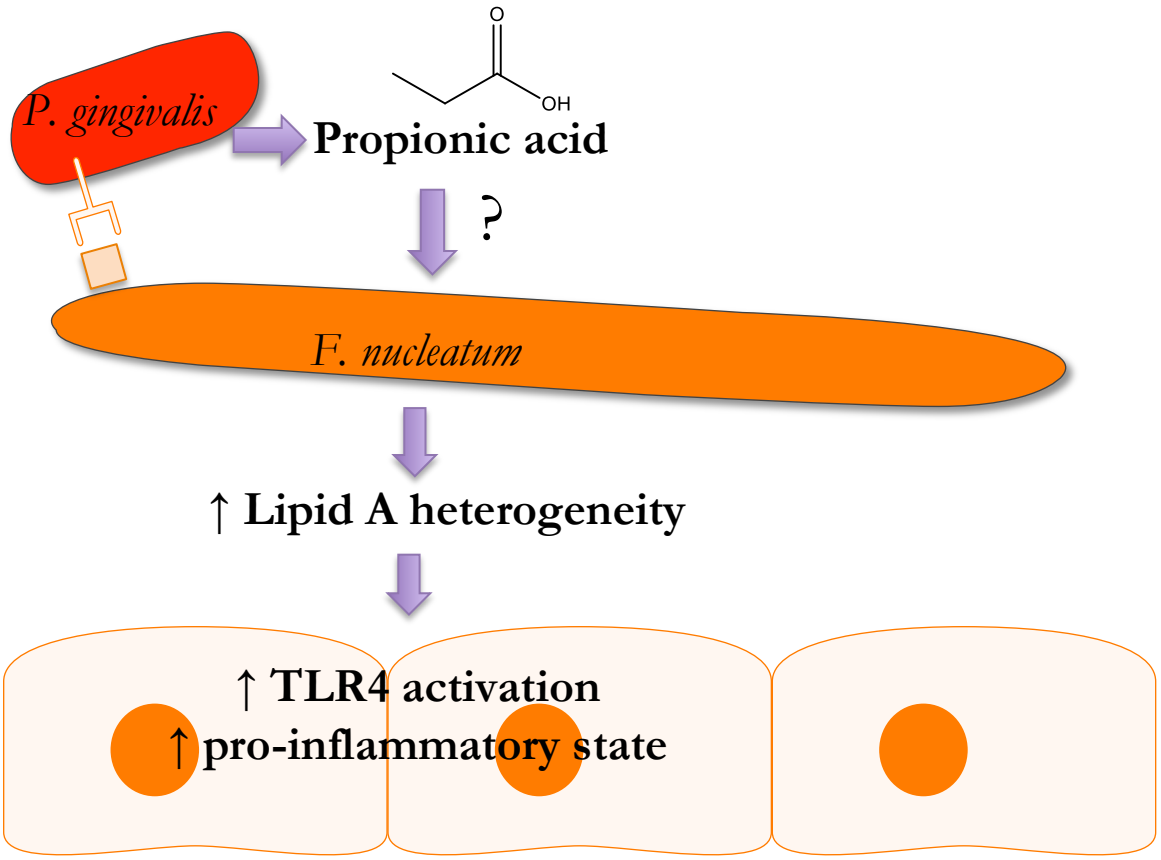


Figure 17. Proposed mechanism for the relationship between P. gingivalis and F. nucleatum and the effect on host innate activation through TLR4.

CHAPTER IV:
F. NUCLEATUM PROPIONIC ACID TRANSPORTER

INTRODUCTION

Although genetic contributions to lipid A heterogeneity has been extensively studied in *E. coli*, *S. typhimirium*, and *P. gingivalis* (Raetz et al., 2009, Wang & Quinn, 2010, Guo *et al.*, 1997), there has yet to be a thorough examination of genes involved in *F. nucleatum* lipid A heterogeneity. Additionally, previous studies, including Aim 2 of this study, have shown that supplementing propionic acid to the media of certain Gram-negative bacteria can elicit increased lipid A heterogeneity (Bainbridge et al., 2008, Ingram *et al.*, 1977) due the relaxed acyl chain specificity of secondary lipid A acyltransferases, LpxA and LpxD. Although uptake of propionic acid is suspected to be necessary for this type of lipid A heterogeneity, the transporters for propionic metabolism relative to lipid A heterogeneity have yet to be characterized. Propionic acid, among other short-chain fatty acids, is a major byproduct of amino acid metabolism by anaerobes *P. gingivalis*, *P. intermedia*, and *F. nucleatum* itself (Lu *et al.*, 2013, Niederman et al., 1997, Singer & Buckner, 1981). We speculated that targeting propionic acid uptake in *F. nucleatum* would affect its ability to modulate its lipid A heterogeneity in the presence of this substrate and affect its interaction with TLR4.

The metabolism of monocarboxylic acids, such as propionic acid, is vital for bacteria to survive in their natural environment. Biosynthesis and degradation of these monocarboxylic acids are well understood, but there is still much confusion about uptake

and transport systems. A propionate-specific transporter has been identified in only one other bacterium and no monocarboxylic acid transporters have been characterized for any *Fusobacterium* species (Kapatral et al., 2002, Chien *et al.*, 2012). Since *F. nucleatum* ATCC 25586 distinctly increases its lipid A heterogeneity in the presence of external propionic acid, as seen in Chapter III, we aim in this chapter to characterize *F. nucleatum* propionate uptake and transport systems through creation of deletion mutants. Lastly, we examine the effect of excess propionic acid on the expression of these putative propionate metabolism genes.

MATERIALS AND METHODS

Bacterial strains

Fusobacterium nucleatum ss. nucleatum strains ATCC 25586 and ATCC 23726 were grown in TYHK media, as described in Chapter II. Electrocompetent *Escherichia coli* DH10b cells, from our culture collection, were prepared using methods previously described (Green & Sambrook, 2012) and grown on Luria-Bertani agar or in broth media.

Creation of ΔFN0814 and ΔFN0815 mutants

F. nucleatum ATCC 25586 genes FN0814 (Accession: NP_603711.1) and FN0815 (Accession: NP_603712.1) were inactivated using a double homologous recombination strategy, similar to one previously described (Coats et al., 2009a). Genomic nucleotide sequences encoding putative propionate permease, FN0815, and putative propionate CoA-transferase, FN0814, were obtained from searches of the annotated F. nucleatum ATCC

25586 genome using analysis tools available with the Integrated Microbial Genomes program (https://img.jgi.doe.gov/cgi-bin/m/main.cgi) (Markowitz *et al.*, 2012). Gene deletions were created by introducing an erythromycin /clindamycin resistance cassette (*ermF-AM*) in place of the coding region for FN0814 or FN0815 (Figure 19). Primer design for PCR amplification of genomic DNA from *F. nucleatum* ATCC 25586 to amplify 700-1000 bp flanking regions upstream and downstream immediately adjacent to the FN0814 and FN0815 coding regions was performed using Primer3Plus program (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Rozen & Skaletsky, 2000). Primers used to amplify these regions are listed in Table 3. PCR amplified flanking regions were co-ligated, with the *ermF-AM* cassette between them, into pcDNA3.1(-) (Life Technologies Inc., Carlsbad, CA, USA) to generate gene disruption plasmids, pFN0814 5'flank: *ermF-AM*: 3'flank and pFN0815 5'flank: *ermF-AM*: 3'flank. These plasmids were electroporated into F. nucleatum ATCC 25586 in a GenePulser Xcell (Bio-Rad, Hercules, CA, USA). Transformed bacteria were plated on TYHK agar plates supplemented with clindamycin (400 ug/ mL) and any mutant colonies arising due to homologous recombination between the flanking segments on the plasmid and the chromosome were selected for further confirmation.

Transcriptional analysis of FN0814 and FN0815

Total bacterial RNA was extracted with the use of TRIzol reagent (Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and then treated with DNAse (Qiagen, Inc., Valencia, CA, USA). To ensure high quality RNA, further purification

was done with RNeasy kit (Qiagen Inc., Valencia, CA, USA). 2 ug of total bacterial RNA was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc, Hercules, CA, USA) following the manufacturer's protocol. Quantitative PCR was performed on a Lightcyler 480 (Roche, Roche Applied Science, Indianapolis, IN, USA), using 1 uL of diluted cDNA to iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc, Hercules, CA, USA) according to the manufacturer's protocol, with primers listed in Table 3. Values presented represent the ratios of expression compared to 16S rRNA expression.

RESULTS

Attempts to construct ΔFN0814 or ΔFN0814 mutants

Although cloning and expansion of disruption plasmids pFN0814 5'flank: *ermF-AM*: 3'flank and pFN0815 5'flank: *ermF-AM*: 3'flank was successful and confirmed by PCR, transformation by electroporation into *F. nucleatum* ATCC 25586 wild-type yielded no confirmed deletion mutants after clindamycin selection at 400 ug/ ml, a concentration recommended by previous reports using similar transformation strategy, albeit for a different subspecies of *F. nucleatum* (Han *et al.*, 2005).

Transcriptional analysis of FN0814 and FN0815

Total cellular RNA was extracted, cDNA synthesized and applied in a quantitative PCR assay to determine transcriptional levels in the presence of excess propionic acid (major

byproduct of anaerobes) or lactic or acetic acids (major byproducts of gram-positive bacteria). Neither of these genes are significantly up-regulated in the presence of propionic acid, suggesting an alternative or secondary transporter or uptake mechanism. However, in the presence of acetic acid, FN0815 is slightly but significantly upregulated relative to untreated growth conditions. This suggests that acetic acid in the environment may also utilize this permease, which may preferentially transport extracellular acetic acid as opposed to propionic acid.

Bioinformatic analysis of FN0815 gene and protein

The propionate permease (*prpP*) gene is most highly characterized from *Ralstonia eutropha* (formerly *Cupriavidus necator*), a Gram-negative soil bacterium which is of keen interest to biotechnology companies since overexpression of this particular gene in *E. coli* yields significant increases in polyhydroxybutyrate and polyhydroxybutyrate-co-hydroxyvalerate. These by-products can be used as a renewable resource to make plastics, as opposed to using crude oil. Growing these bacteria in the presence of propionic acid increases both the diversity and overall production of these two bio-based polymers, thus suggesting the critical role of propionic uptake by PrpP (Chien et al., 2012, Horng *et al.*, 2013).

To evaluate propionic transporters in *F. nucleatum* strains, *R. eutropha* prpP (Accession: WP_013958009) was BLAST searched against several complete *F. nucleatum* genomes, of which resulted in three top hits with putative proteins from: *F. nucleatum* ATCC

25586 (FN), *F. nucleatum* ATCC 49526 (FNV), and *F. nucleatum* subsp polymorphum 12230 (FN12230). All four sequences encode a membrane protein, with at least 9 highly conserved transmembrane helices (Figure 21).

We also analyzed the aligned amino acid sequences by obtaining a percent identity matrix, seen in Figure 22, also using Clustal Omega tools (Sievers et al., 2011). Although functional similarity is relatively lower between sequences from *R. eutropha* and all *F. nucleatum* strains, those from *F. nucleatum* maintain strongly similar identity, suggesting that this putative transporter is highly conserved across *F. nucleatum* subspecies.

DISCUSSION

Although a specific active transporter for *F. nucleatum* was not successfully characterized, this bacterium may still be capable of utilizing other pathways for propionate uptake. Transcriptional analysis demonstrated that neither suspected propionate metabolism genes, FN0814 nor FN0815, were up-regulated when F. nucleatum was grown in the presence of excess propionic acid. Despite this observation, the bacterium was still capable of modulating lipid A diversity in response to propionic acid resulting in increased potency at TLR4.

An active, secondary propionate transporter has been identified for *Corynebacterium glutamicum*, a soil bacterium used for biotechnology production and bioremediation. This secondary transporter, MctC, belongs to a class of sodium solute symporters, but is driven by an electrochemical proton potential. Of three substrates, this protein was shown to have

the greatest affinity for propionate and acetate, and much lower affinity for pyruvate (Jolkver *et al.*, 2009). Possibly, *F. nucleatum* may possess multiple active transport systems, which, like MctC, may be highly regulated and respond to changes in substrate availability. Regardless of the transporter system, it is still unclear how specifically these pathways feed into the LPS biosynthetic pathway, or "Raetz pathway" (Raetz et al., 2009, Wyckoff et al., 1998), and thus demand further inquiry.

Further complicating this study was the inherent difficulty to genetically manipulate *F. nucleatum*, particularly the ATCC 25586 type strain. There have been a limited number of genetic deletion studies in *F. nucleatum* compared to other oral bacteria, such as *P. gingivalis*. This is likely due to the presence of many native restriction endonucleases with four-base recognition sites and severe restriction modification systems consisting of methylated DNA in the *F. nucleatum* genome, increasing difficulty of genetic alteration by transformation in this species (Kinder Haake *et al.*, 2006, Roberts *et al.*, 2005, Lui *et al.*, 1979). Furthermore, it is not known whether functionality of the propionate permease is critical for the viability of the microorganism. Previous cloning studies have either only overexpressed *prpP* gene in *E. coli* for production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate biopolymer (Horng et al., 2013, Liu *et al.*, 2009) or examined downstream propionate metabolic genes in the *prpBCDE* operon in other bacteria (Lee & Keasling, 2005). Therefore our attempt to disrupt the FN0814 and FN0815 *(prpP)* genes may have resulted in a lethal mutation, thus resulting in the lack of viable transformants. Future studies should explore more genetically tractable clonal types belonging to the same subspecies, nucleatum, identify alterations to lipid A and

host response. A recent study employed, for the first time, a *F. nucleatum* ATCC 23726 genetic library by transposon mutagenesis (Coppenhagen-Glazer *et al.*, 2015). Over 1,200 clones were screened in 96-well format for hemagglutination deficiency, of which three mutants were identified. A similar strategy could be used to screen *F. nucleatum* ATCC 23726 transposon mutants grown in TYHK medium supplemented with propionic acid. In a 96-well format, the mutants could be rapidly screened for increased TLR4 activation in our dual-luciferase reporter assay.

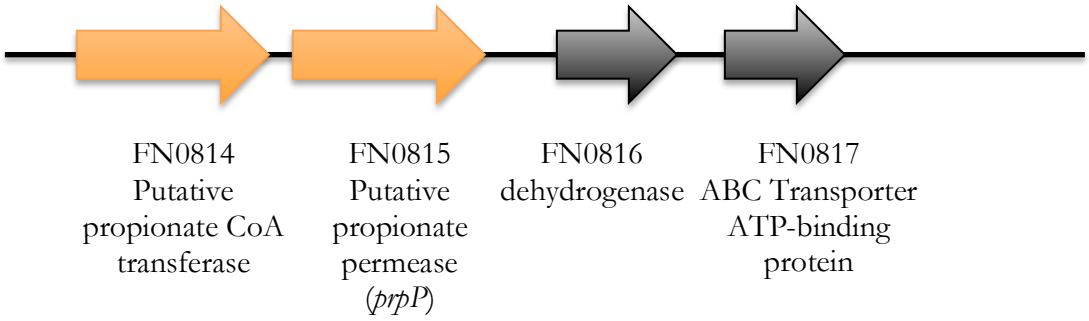


Figure 18. Putative propionate metabolism genes in *F. nucleatum* ATCC 25586 genome identified by BLAST analysis. FN0814 and FN0815 (in gold) were putatively identified as genes which may contribute to specific uptake and transfer of propionate intracellularly.

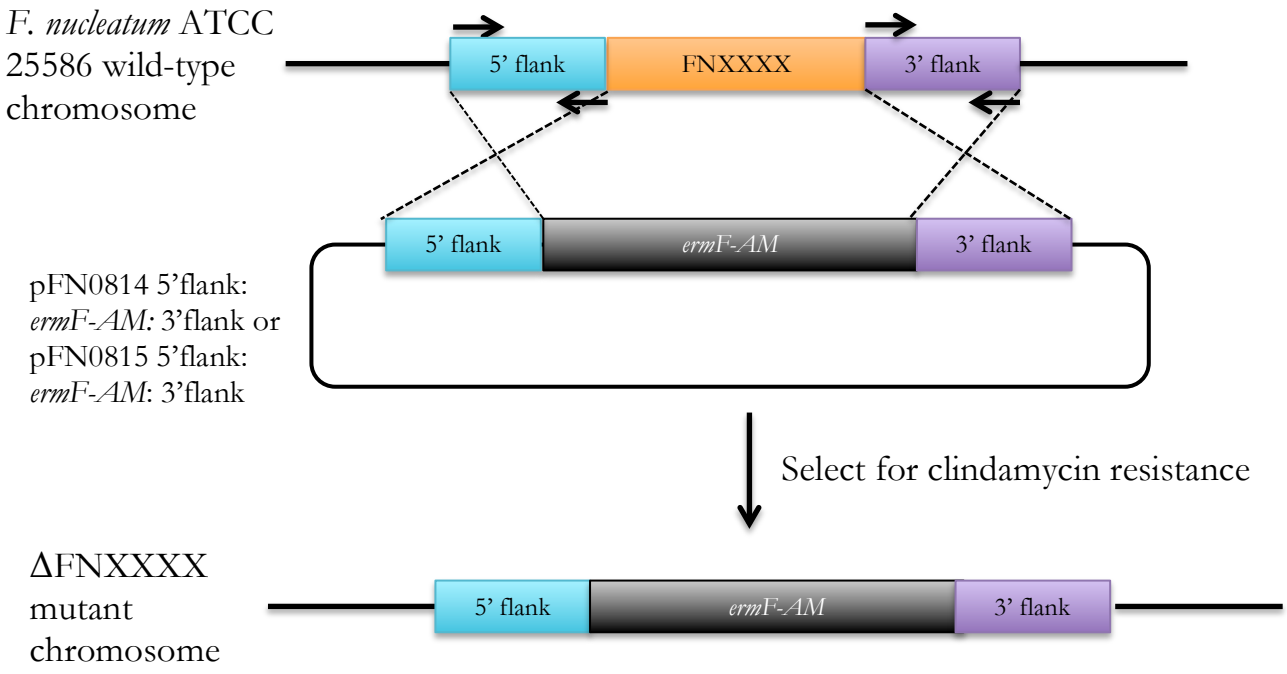


Figure 19. Schematic representation of the construction of ΔFN0814 or ΔFN0815 mutants. Genetic deletions were created by introducing erythromycin/clindamycin resistance cassette, *ermF-AM*, in place of the coding region for the genes of interest.

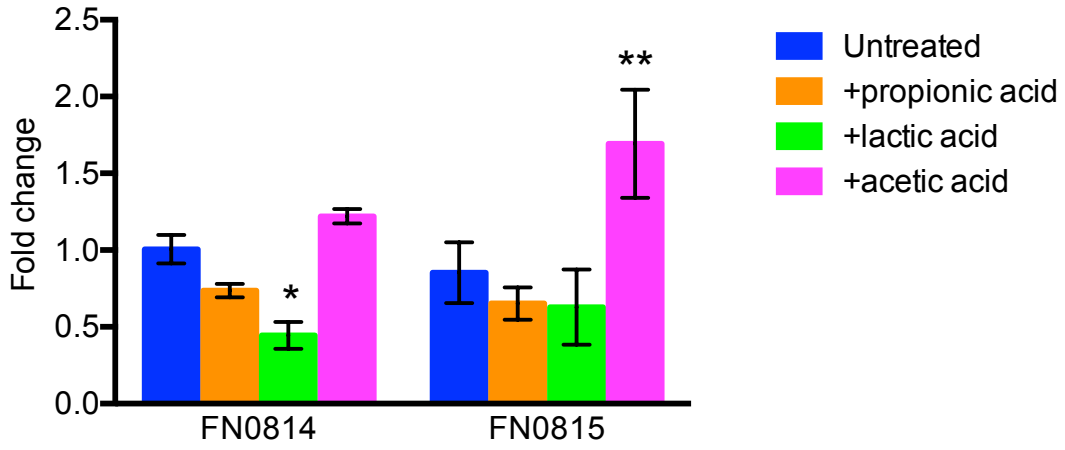


Figure 20. Transcriptional expression analysis of FN0814 and FN0815 by quantitative PCR. Total cellular RNA was extracted, cDNA synthesized and applied in a quantitative PCR assay to determine transcriptional levels in the presence of excess propionic acid (major byproduct of anaerobes) or lactic or acetic acids (major byproducts of gram-positive bacteria) Neither of these genes are significantly up-regulated in the presence of propionic acid, suggesting an alternative or secondary transporter or uptake mechanism. However, in the presence of acetic acid, FN0815 is slightly but significantly upregulated relative to untreated growth conditions. This suggests that acetic acid in the environment may also utilize this transporter.

Table 3. Oligonucleotide sequences used for cloning and transcriptional analyses for Chapter IV.

Primers	Sequence (5'→3')	Product size (base pairs)
FN0814-5'Flank		
Forward	AGTACGAATTCTGATTATCTAGAGGAGTT	700
Reverse	CATGAGTCGACAGGTTTGCATGAAGACTACG	
FN0814-3'Flank		
Forward	ACTAGACGCGTCTTATGTATCTTGCTTAT	970
Reverse	TGCACGCTAGCGCAAGTCCAGCAATTACAT	
FN0815-5'Flank		
Forward	AGTACGAATTCGGTATTCATATAGCTTCAGCC	1100
Reverse	CATGAGTCGACGCAAGATACATAAGC	
FN0815-3'Flank		
Forward	ACTAGACGCGTGGTATGTGTACTGCTATTATCCCC	1000
Reverse	TGCACGCTAGCAACAGACACTACTGGT	
ermF-AM		
Forward	ATGCGTCGACCATCGGTATTTGCAACATC	2100
Reverse	GCATACGCGTCCGAAGCTGTCAGTAGTAT	
FN0814-1		
Forward	ATTTTTGCAGCTGGATTTGG	105
Reverse	TGGAGCTAGTCCCCAATGTC	
FN0815-2		
Forward	GGACTTGCAGCATTTGCTATT	105
Reverse	GCCCGTTATACCAGCAAGAG	
16s rRNA		
Forward	CAGCTCGTGTCGTGAGATGT	101
Reverse	TCGTAGGCAGTATCGCATGA	

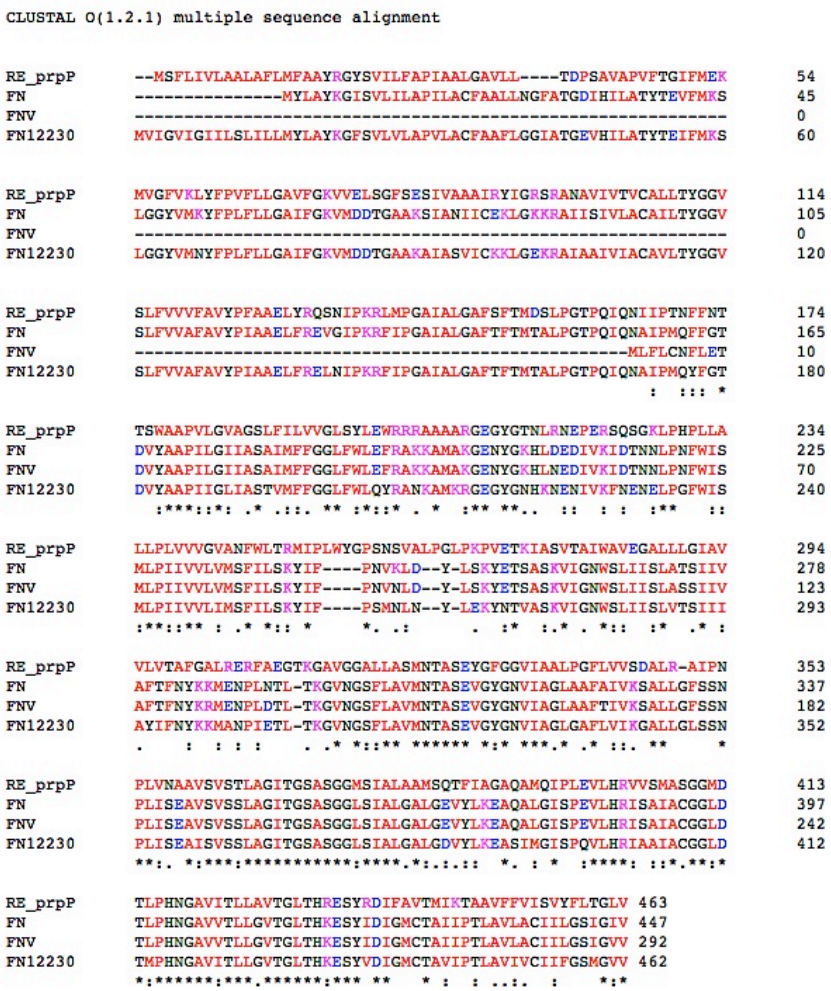


Figure 21. Multiple sequence alignment of propionate permease amino acid sequence (PrpP) from *R. eutropha* (RE) with *F. nucleatum* proteins bearing strong homology. RE_prpP was BLAST searched against several complete *F. nucleatum* genomes, of which resulted in three top hits with putative proteins from: *F. nucleatum* ATCC 25586 (FN), *F. nucleatum* ATCC 49526 (FNV), and *F. nucleatum* subsp polymorphum 12230 (FN12230). All four sequences encode a membrane protein, with at least 9 highly conserved transmembrane helices. Asterisks (*) below alignments denote a single reserved residue, colons (:) denote residues with strongly similar properties, and semi-colons (;) denote weakly similar properties (Sievers et al., 2011)

	RE_prpP	FN	FNV	FN12230
RE_prpP	100	47.74	41.92	45.27
FN	47.74	100	94.86	82.55
FNV	41.92	94.86	100	78.08
FN12230	45.27	82.55	78.08	100

Table 4. Percent identity matrix of amino acid sequence aligned using Clustal Omega program (Sievers *et al.*, 2011). Alignment score for pairwise comparisons of sequences aligned in Figure 21. Although functional similarity is relatively lower between sequences from *R. eutropha* and all *F. nucleatum* strains, those from *F. nucleatum* maintain strongly similar identity, suggesting that this putative transporter is highly conserved across *F. nucleatum* subspecies.

CHAPTER V:
SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The preceding chapters have revealed the role of the oral polymicrobial community on the activation of TLR4. In chapter II, matched subgingival plaque collected from healthy diseased sites demonstrated a diverse range of TLR4 activity. Diseased sites activated very strongly, whereas healthy sites demonstrated strong antagonism in addition to mild antagonism or agonism and strong agonism. This behavior has not been previously demonstrated by a clinical sample. In addition, TLR4 activation was found to be highly associated with clinical measurements of disease, particularly probing depth. In chapter III, specific polymicrobial interactions with *F. nucleatum,* a member important for the development and maturation of the community, was explored and evaluated for lipid A-TLR4 structure-function alterations. In the presence of periodontopathogen, *P. gingivalis* and its metabolic by-product, propionic acid, *F. nucleatum* not only increases its lipid A composition, but also activates TLR4 more potently than in mono-culture. Lastly, Chapter IV attempted to demonstrate the role of a propionic transporter. Transcriptional analysis demonstrates that annotated propionate metabolism genes in the ATCC 25586 genome are not regulated by the presence of an extracellular source of propionic acid, indicating that other propionic acid uptake mechanisms may be involved upstream of *F. nucleatum* lipid A modification. Furthermore, these genes could be temporally regulated as well and more analyses are needed.

It is estimated that nearly half the adult population in the United States have mild to severe periodontitis, while the prevalence rate is 70 percent for adults age 65 or older (Eke *et al.*, 2012). Additionally, inflammation as a result of chronic periodontal disease may present as a risk factor for systemic complications such as diabetes, pre-term low birth weight, cardiovascular disease, rheumatoid arthritis, and obesity (Lakschevitz *et al.*, 2011, Han et al., 2004, Tonetti *et al.*, 2013, Scher *et al.*, 2014, Chaffee & Weston, 2010, Hajishengallis *et al.*, 2002). While the etiology of the disease is strongly associated with the presence of a microbial community and although there has been substantial investigation towards the characterization of virulence factors in specific bacteria, there is limited understanding about how a polymicrobial community in health and disease engages the host and elicits inflammation. Understanding how the microbial community changes during periodontal health to disease can reveal possible targets for intervention. Moreover, the monitoring of subgingival plaque in a periodontal site and measurement of its TLR4 activation may help to determine future development to disease and targeting of biosynthetic pathways of virulence factor, lipid A, may be a diagnostic indicator of destructive sites before bone and tissue loss occurs.

FUTURE DIRECTIONS

Identification of TLR4 antagonistic sources and further in vivo *characterization*

Although healthy plaque samples are capable of antagonizing TLR4 activation, it is unclear what precisely are the sources and whether these sources are derived from the host, the polymicrobial community, or from both acting synergistically. If the source of TLR4

antagonism is bacterial in origin, there is a possibility that low-abundance Gram-negative species, such as *P. gingivalis*, may produce TLR4-antagonistic lipids similar to those seen *in vitro* structural studies (Coats et al., 2005, Darveau *et al.*, 1998). Although the work described in these chapters also demonstrates *in vitro* characterization of structural modifications and modulation of TLR4 signaling, identification of potential TLR4-antagonistic molecules directly from patient samples could reveal major insights into structure-function relationships between microbes and the host. The use of *in vitro* bacterial culture provides yields large enough and pure enough for structural characterization. However, these conditions still represent artificial conditions as opposed to physiologically and medically relevant conditions.

Currently, there is no effective procedure to purify lipids directly from patient samples for structural characterization by mass spectrometry. This may be due to limitations in the sample size or presence of compounds which interfere with ionization and detection, such as salts and detergents. A future study could employ and modify recent lipid extraction methodologies from isolated bacterial colonies which eliminate addition of external detergent sources and which extract sufficient quantities for structural and potentially functional characterization (El Hamidi *et al.*, 2005, Zhou *et al.*, 2009). In addition to front end optimization of sample preparation, future studies could improve upon back end ionization and mass detection, such as utilization of novel surface acoustic wave nebulization in lieu of electrospray or MALDI (Yoon *et al.*, 2012) and use of an automated lipid-specific structure assignment algorithm (Ting *et al.*, 2011). A major limitation to these studies may be due to

contaminating host molecules found in the patient samples. We have provided in this thesis one modification to MALDI matrix preparation to enhance peak detection (Figures 51 and 52). Nonetheless, detection of the source of antagonism, whether host or bacterial-derived, will be critical for understanding the underlying mechanisms involved with dampening TLR4 signaling in a healthy site.

Short-chain fatty acids in the periodontium

Short-chain fatty acids (SCFAs), such as acetic, propionic, and butyric acid, are metabolic by-products of fermentation by anaerobic bacteria. These monocarboxylic acids are found in found in high levels in the oral cavity of patients with periodontal disease, in the genital tract of women with bacterial vaginosis, and also in the gut (Niederman et al., 1997, Chaudry *et al.*, 2004, Wong *et al.*, 2006). Although described as having potentially beneficial for intestinal homeostasis(Tan *et al.*, 2014), SCFAs produced by resident fermentative bacteria could play a role in the development of disease in the periodontium (Stehle et al., 2001).

Studying how SCFAs affect both the microbiota and the host could reveal major metabolic relationships within and between the two. The work described here demonstrates that propionic acid could contribute to a dysbiotic microbiota by being utilized by *F. nucleatum* to modify its lipid A composition. This modification leads to an increase in TLR4 activation and thus promotes a pro-inflammatory state in the host (Figure 17). Other studies have also described the role of SCFAs in modulation of microbe-host interaction. A recent

study demonstrated that SCFAs not only induce multiple epigenetic changes to reactivate latent Kaposi's sarcoma –associated herpesvirus and HIV, but are also able to activate other host machinery, such as RNA polymerase II, for effective transcription of viral genes (Yu et al., 2014). Future work would couple gas-chromatography mass spectrometry, to quantitate and track changes in SCFA composition in subgingival plaque and crevicular fluid, and functional analysis using assays such as the dual-luciferase reporter assay described in this work. Additionally, more work to evaluate additional SCFAs on *F. nucleatum* lipid A heterogeneity would further reveal

Understanding function at the transcriptomic level

Although the work described in Chapter IV did not result in the creation of successful deletion mutants, transcriptional analysis by qPCR did provide interesting insight into the response of these particular annotated genes. However, use of qPCR alone fails to capture the overall picture of what genes and pathways are upregulated in a polymicrobial community. Very recently, researchers from the Forsyth Institute conducted metatranscriptome analysis on subgingival samples from progressing and stable sites from periodontitis patients. Using Next Generation Sequencing, community-wide expression profiles revealed not only upregulation of virulence genes by periodontopathogens such *P. gingivalis* and *Tannerella forsythia*, but also by species not classically associated with disease, such as *S. gordonii, Veillonella* and *S. oralis* (Yost et al., 2015*)*. Future studies could utilize comparative RNAseq analysis of *F. nucleatum* grown in mono-culture and in co-culture with

other bacteria, such as *P. gingivalis, S. gordonii, Veillonella* and *Tanerella forsythia.* With the results of the recent meta-RNAseq analyses of subgingival plaque, using this more specific analysis could identify alterations in not only *F. nucleatum,* but the other bacteria in the community with which it associates.

In closing, it is clear that utilizing only one level of analysis will not be sufficient for future understanding of such a complex microbiota and its relation to its equally complex host anatomical surroundings. Periodontal disease is not an infectious disease in Koch's classical definition, lacking a defined causative agent. Therefore, application of a wide breadth of tools - such as cellular assays, molecular biology, mass spectrometry, and Next Generation Sequencing - simultaneously is necessary for elucidating the mechanisms for how this disease develops initially and remains a chronic condition, leading to potential targets for intervention or preventative therapies.

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Kaposi's Sarcoma-Associated Herpesvirus Replication. *Journal of Virology* **88**: 4466-4479.

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CURRICULUM VITAE

Thao (Jenny) To

Department of Periodontics, University of Washington, Box 357444, Seattle, WA 98195
206-616-2614 (office), thao2@uw.edu

EDUCATION

PhD, **Oral Biology**, July 2015
University of Washington
Research advisor: Richard Darveau, PhD

BSc, Microbiology, June 2008
University of Washington

TEACHING EXPERIENCE

Teaching Assistant, University of Washington
ORALB 520: Medical Microbiology, Spring 2015

- Created syllabus and learning objectives
- Lectured on "General Bacteriology and Enteric Bacteria" and "Immunity and Vaccinology"
- Maintained gradebook and course website through LearningWare
- Prepared online quizzes and final exam

Teaching Assistant, University of Washington
ORALB 521: Molecular Microbiology and Oral Diseases, Fall 2014

- Lectured on "Introduction to the Microbiology of Periodontal Disease" to 1st year dental students
- Graded weekly short-answer quizzes
- Maintained gradebook and course website through LearningWare
- Prepared midterm and final exams

Teaching Assistant, University of Washington
ORALB 580: Molecular Biology Laboratory, Fall 2014

- Assisted in lab instruction of graduate Oral Biology students
- Supervised lab methods: DNA/RNA extraction, PCR, Western blot, Southern blot, cloning into plasmid vectors

RESEARCH EXPERIENCE

Graduate Research Trainee, University of Washington, 06/2010-present

- Characterized the inflammatory potential of subgingival plaque samples from periodontitis patients using *in vitro* system measuring Toll-like receptor 4 activation
- Characterized structure of lipid A from *Fusobacterium nucleatum* using MALDI-TOF mass spectrometry
- Functionally characterized *F. nucleatum* synergistic action with other oral bacteria on periodontal inflammation
- Developed novel MALDI-TOF matrix formulation for enhanced detection of highly heterogenerous lipid mixtures

Research Scientist/ Engineer I, University of Washington, 2008-2010

- Developed chromatography separation method of *Porphyromonas gingivalis* lipid A variants for quantitation
- Trained and specialized in oral anaerobic bacterial culture and identification
- Performed chemical elucidation of unknown lipid A structures using MALDI-TOF tandem mass spectrometry
- Trained and supervised undergraduate students and visiting scientists in diverse methods: LPS/lipid A isolation, anaerobic bacterial growth, ELISA assays, and mass spectrometry

UW-HHMI Biology Undergraduate Research Intern, University of Washington, 2006-2008

- Screened genetic library of *P. gingivalis* lipid A phosphatase mutants for cationic antimicrobial peptide susceptibility
- Assisted in transposon mutagenesis of *P.gingivalis* suspected lipid A phosphatase genes

RESEARCH FUNDING

1. 2012/07/01-2017/06/30
   T90 DE021984-01A1, National Institute of Dental & Craniofacial Research (NIDCR)
   Derouen, Timothy A and Ramsay, Douglas (PI)
   Comprehensive Training in Inter-Disciplinary Oral Health Research

2. 1982/07/01-2012/06/30
   T32 DE007132-28, National Institute of Dental & Craniofacial Research (NIDCR)
   Derouen, Timothy A (PI)
   Comprehensive Training in Inter-Disciplinary Oral Health Research

RESEARCH COLLABORATIONS

UW Applied Physics Laboratory and Biolase, Inc, 12/2014-3/2015

- Professional technical consulting
- Developed assay to evaluate bioeffect of pulsed lasers on disruption of dental microbial viability and growth
- Developed novel enriched growth medium for expedited pigmentation by *Porphyromonas gingivalis*
- Performed bacterial viability studies and analyzed results

David Goodlett group, Medicinal Chemistry, University of Maryland-Baltimore, 09/2010-06/2011

- Rotation project: characterized and separated bioactive anti-inflammatory molecules from cocoa extracts using high-resolution UPLC-MS/MS
- Work presented at ASMS 2011 and contributed to NIH-SBIR grant submission (Theo Chocolate)

Michael Curtis group, Queen Mary University of London, UK, 07/2010-present

- Isolated and characterized novel fecal and gastrointestinal lipids
- Work resulted in manuscript currently in preparation

Thomas Mitchell group, University of Louisville, 2010-2013

- Isolated and structurally characterized monpohosphoryl lipid A variants for use as vaccine adjuvants
- Work resulted in publication

PUBLICATIONS

To TT, Gumus P, Nizam N, Buduneli N, Darveau RP. Subgingival plaque in periodontal health antagonizes at TLR4 and inhibits E-selectin expression on endothelial cells. Infection and Immunity (Manuscript submitted #IAI00693-15)

Coats SR, Paramanov NA, To **TT**, Curtis MA, Darveau RP. Intestinal dysbiosis exacerbates TLR4 signaling via disruption of a host lipid barrier to LPS. (Manuscript in preparation)

Chilton PM, Hadel DM, **To TT** , Mitchell TC, Darveau RP. Adjuvant activity of naturally-occurring monophosphoryl lipid A preparations from mucosal-niche bacteria. Infect Immun. 2013; 81(9):3317-25. PMCID: 3754217

Coats SR, Berezow AB, **To TT**, Jain S, Bainbridge BW, Banani KP, Darveau RP. The lipid A phosphate position determines differential host Toll-like receptor 4 responses to phylogenetically related symbiotic and pathogenic bacteria. Infect Immun. 2011;79(1):203-10. PMCID: 3019871.

Coats SR, **To TT**, Jain S, Braham PH, Darveau RP. *Porphyromonas gingivalis* resistance to polymyxin B is determined by the lipid A 4'-phosphatase, PGN_0524. Int J Oral Sci. 2009;1(3):126-35. PMCID: 2909122.

Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, **To TT**, Goodlett DR, Ernst RK, Darveau RP. Human Toll-like receptor 4 responses to *P. gingivalis* are regulated by lipid A 1- and 4'-phosphatase activities. Cell Microbiol. 2009;11(11):1587-99. PMCID: 3074576.

PRESENTED ABSTRACTS

To TT, Coats SR, Sadikin RA, Darveau RP. Polymicrobial communities modify *Fusobacterium nucleatum* lipid A structure-function relationships in periodontal innate immunity [Abstract and poster]. Presented at PgLondon: 2nd International Conference on *Porphyromonas gingivalis* and related species in oral and systemic diseases, London, United Kingdom. June 2015.

To TT, Coats SR, Sadikin RA, Darveau RP. Polymicrobial communities modify lipid A structure-function relationships in periodontal innate immunity [Abstract and oral presentation]. Presented at 1st American Society for Microbiology Conference on Polymicrobial Infections, Washington, D.C., November 2014.

To TT, Gümüş P, Nizam N, Buduneli N, Darveau RP. Toll-like Receptor 4 and 2/1 Activation by Subgingival Plaque [Abstract and poster]. Presented at Penn Periodontal 2013 Conference, Philadelphia, PA, June 2013.

To TT, Mackay CL, Sadilek M, Langridge-Smith P, McShea A, Darveau RP, Goodlett DR. Profiling of bioactive compounds in chocolate extracts by high resolution mass spectrometry [Abstract and poster]. Presented at 59th Annual Conference of the American Society of Mass Spectrometry, Denver, CO, June 2011.

To TT, Coats SR, Bainbridge BW, Darveau RP. Characterization of a 4'-Lipid A Phosphatase in Oral Pathogen, *Porphyromonas gingivalis*. [Abstract and poster] UW School of Dentistry Research Day and UW-HHMI Research Day, September 2007.

AWARDS

- American Society for Microbiology Student Travel Award, 2014
- Graduate School Fund for Excellence and Innovation Graduate Student Award, 2014
- NIH T90 Predoctoral Training Fellowship, 2012-15
- NIH T32 Predoctoral Training Fellowship, 2010-11
- UW-HHMI Biology Undergraduate Research Internship, 2006-07
- UW-HHMI Biology Undergraduate Fellowship 2004-05
- Philips Medical Systems Scholarship, 2004-08

- Providence General Children's Association Scholarship, 2004
- Jill L. Renshaw Human Services Scholarship, 2004-06
- UW Mary Gates Honors Scholarship, 2004-06

INVITED MANUSCRIPT REVIEWER

- Mediators of Inflammation

AFFILIATIONS

- Member, American Society for Microbiology 2014-present
- Member, Association for Women in Science, 2013-present
- Member, American Association for the Advancement of Science, 2011-present
- Member, American Society for Mass Spectrometry, 2010-present

LEADERSHIP AND COMMUNITY SERVICE

- Support Volunteer, Public Health Reserve Corps of King County, 2015-present
- Dental Clinic Support Volunteer, Seattle/ King Country Clinic with Remote Area Medical, 2014-present
- Oral Biology Senator and Travel Grants Committee Member, UW Graduate and Professional Student Senate, 2010-2012
- Science and math tutor for Somali refugee high-school students, Secondary Bilingual Orientation Center, Seattle, WA, 2008-10