Title
Characterization of Treponema denticola Pathogenic Potential in a Periodontal Disease Setting; Cytoskeletal and Intracellular Dynamics

Author
Malone, Erin

Publication Date
2023

Characterization of Treponema denticola Pathogenic Potential in a Periodontal Disease Setting; Cytoskeletal and Intracellular Dynamics

by
Erin Malone

DISSERTATION
Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Oral and Craniofacial Sciences

in the

GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Approved:

DocuSigned by:
DC1E271FE8F947A...

Ling Zhan
Chair

DocuSigned by:
Yvonne L. Hernandez-Kapila
D...4D8...

Yvonne L. Hernandez-Kapila

DocuSigned by:
Alice Goodwin
C84D7533F9714EF...

Alice Goodwin

Committee Members

1.1.1 Dedication and Acknowledgments

I dedicate this work to those who frame my support system. I dedicate this work to my God, my wife Robyn Malone, my daughter Quinn Malone, and our baby to be. I also dedicate this work to my father Ernie Malone, mother Kim Malone, and all my family members. Thank you for being my motivation to move forward. Thank you for giving me strength when I had no more energy to give. Thank you for believing in me when I did not believe myself. Thank you for loving me, despite my shortcomings. I dedicate all my success to you.

I dedicate this work to my mentors Dr. Yvonne Kapila, DDS, Phd. Thank you taking a chance and accepting me when every other lab doubted my capabilities. Thank you for your support and being the example for my career that I strive to attain.

I dedicate this work to my lab mates and classmates, notably Sean Ganther and Allan Radaic. Thank you for being encouraging voices and a sound board to bounce ideas which directed all the following ideas.

I dedicate this work to my collaborators, thesis committee members, and my interim mentor, Dr. John C. Fenno, Dr. Ling Zhan, and Dr. Alice Goodwin. Thank you for your creative ideas and resources used in all of these experiments. Thank you for guiding my research which contributed to my success as a researcher.

1.1.2 Contributions

Malone, E. T., Ganther, S., Mena, N., Radaic, A., Shariati, K., Kindberg, A., Tafolla, C., Kamarajan, P., Fenno, J. C., Zhan, L., & Kapila, Y. L. (2021). *Treponema denticola*-Induced RASA4 Upregulation Mediates Cytoskeletal Dysfunction and MMP-2 Activity in Periodontal Fibroblasts. *Frontiers in cellular and infection microbiology*, *11*, 671968. https://doi.org/10.3389/fcimb.2021.671968

Ganther, S., Radaic, A., **Malone, E.,** Kamarajan, P., Chang, N. N., Tafolla, C., Zhan, L., Fenno, J. C., & Kapila, Y. L. (2021). *Treponema denticola* dentilisin triggered TLR2/MyD88 activation upregulates a tissue destructive program involving MMPs via Sp1 in human oral cells. *PLoS pathogens*, *17*(7), e1009311. https://doi.org/10.1371/journal.ppat.1009311

Nguyen, T., Sedghi, L., Ganther, S., **Malone, E.,** Kamarajan, P., & Kapila, Y. L. (2020). Host-microbe interactions: Profiles in the transcriptome, the proteome, and the metabolome. *Periodontology 2000*, *82*(1), 115–128. https://doi.org/10.1111/prd.12316

Radaic, A., **Malone, E**., Kamarajan, P., & Kapila, Y. L. (2022). Solid Lipid Nanoparticles Loaded with Nisin (SLN-Nisin) are More Effective Than Free Nisin as Antimicrobial, Antibiofilm, and Anticancer Agents. *Journal of biomedical nanotechnology*, *18*(4), 1227–1235. https://doi.org/10.1166/jbn.2022.3314

Radaic, A., Ye, C., Parks, B., Gao, L., Kuraji, R., **Malone, E.,** Kamarajan, P., Zhan, L., & Kapila, Y. L. (2020). Modulation of pathogenic oral biofilms towards health with nisin probiotic. *Journal of oral microbiology*, *12*(1), 1809302. https://doi.org/10.1080/20002297.2020.1809302

Abstract

Characterization of *Treponema denticola* pathogenic potential in a periodontal disease setting; cytoskeletal and intracellular dynamics - Erin Malone

Periodontal disease is characterized by destruction of the hard and soft tissues that comprise the periodontium. This destruction translates to a degradation of the extracellular matrices (ECM), which is mediated by bacterial proteases, host-derived matrix metalloproteinases (MMPs), and other proteases released by host tissues and immune cells. Bacterial pathogens interact with host tissue and thereby trigger adverse cellular functions, including a heightened immune response, tissue destruction, and tissue migration. Oral spirochete, *Treponema denticola* (*Td*), is highly associated with periodontal disease. A *Td* outer membrane protein complex called dentilisin, contributes to the chronic activation of pro-MMP-2 in periodontal ligament (PDL) cells and triggers increased expression levels of activators and effectors of active MMP-2 in PDL cells. Despite these advances, there is no mechanism known for dentilisin-induced MMP-2 activation, PDL cytopathic behaviors leading to disease, or potential treatment. This thesis demonstrated that *Td* through dentilisin influences PDL cellular functions such as metabolism, contractility, migration, actin cytoskeletal reorganization, and mechanotransduction. Furthermore, we show dentilisin through an actin-RASA4-MMP-2 axis influences depolymerization and over expression and activity of MMP-2. Therapeutically, one possible avenue to combat these interactions is through the use of bacteriocin nisin. This thesis demonstrated that nisin delivered in a solid lipid nanoparticle (SLN-Nisin) is an effective anti-microbial against *Td,* with the potential to bacterial clearance inside and outside of the PDL cells. Proper clearance of *Td* may provide a more symbiotic microbiome and “reset” homeostatic conditions needed for repair, remodeling, or regeneration of the periodontium. Thus, the main hypotheses of this proposal are that *T. denticola* interactions with PDL cells mediate adverse effects on homeostasis and cellular functions leading to a compromised cellular phenotype. Additionally, nisin is a potential therapeutic for abrogating these effects. This hypothesis was tested using various functional assays, live imaging dyes and immunofluorescent staining via confocal microscopy, RNA sequencing and real-time quantitative polymerase chain reaction, western blotting, bacterial mutants, and functional gene knockdown approaches. The following dissertation provides molecular evidence for a mechanism of *Td*-mediated adverse cytopathic effects leading to cellular characteristics consistent with severe periodontitis; and evidence for nisin as a potential therapeutic for reducing or reversing *Td*-mediated periodontopathic processes in vitro.

Table of Contents

List of Figures

List of Abbreviations

Abbreviation	Full Name
Td or *T. denticola*	*Treponema denticola*
PD	Periodontal Disease
PDL	Periodontal Ligament
hPDL	Human Periodontal Ligament
MMP	Matrix Metalloproteinases
SLN	Solid Lipid Nanoparticle
SLN-Nisin	Solid Lipid Nanoparticle containing Nisin Complex
Col	Collagen
RASA4	RAS P21 Protein Activator 4
Jasp	Jasplakinoide
LatB	Latrunculin B
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
CD or CytoD	Cytochalasin D
ECM	Extracellular Matrix
Cal	Clinical Attachment Loss
Pg or P. gingivalis	*Porphymonas gingivalis*
FN or F. nucleatum	*Fusobacterium Nucleatum*
Tf or T. Forsythia	*Tannerella Forsythia*
AA or A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*

INTRODUCTION

The periodontal ligament is responsible for regeneration of the periodontium including restoration of the ligament, extracellular matrix, alveolar bone, peripheral nervous system, cementum, and even endothelial cells. With such a dynamic multipotent tissue there is no wonder why disturbance of its function can lead to detrimental consequences. The most documented and characterized pathologic consequence that effects all listed tissues is described as periodontal disease. Classically, periodontal disease is initiated by a microbial infection which stimulates a persistent inflammatory response. Over time, the inflammatory state transitions from a healthy periodontium to gingivitis. As the microbial pathogens remain and calculus builds, epithelial, gingival tissues loose attachments and expose the periodontal ligament which connects the alveolar bone to the tooth root cementum. The tipping point of disease progression from gingivitis to periodontal disease seems to be the loss of the periodontal ligament attachment. At homeostasis the PDL is essential in distributing mechanical load back and forth between the tooth to the alveolar bone influencing bone remodeling, maintaining stability of the tooth within the alveolar bone socket, matrix turnover, recognition of foreign pathogens through inflammatory signaling, and regeneration of the periodontium. In the context of periodontal disease, bacterial pathogens cloud the proper functioning of the periodontal ligament and its active signaling fibroblasts. Pathogens illicit chronic inflammatory responses through the PDL leading to prolonged inflammatory mediators that destroy the extracellular matrix and host tissue destruction in hopes to clear the infection. Loss of the PDL increases tooth mobility and the amount of irreversible clinical attachment loss (CAL) which are both key clinical indicators for staging and grading patients compromise by periodontitis. Additionally, loss of the PDL diminishes the ability for the periodontium to replenish itself through regenerative pathways. Because of these truths, the scientific world has an invested interest in maintaining, protecting,

and regenerating the periodontal ligament as it is an essential regulator of homeostasis and disease.

The most abundant cell type of the PDL is periodontal ligament fibroblasts. A key responsibility of these cells is the production and maintenance of the extracellular matrix. By maintaining attachments from cell-to-cell contact and attachments to the extracellular matrix, the PDL cell can constantly sense changes and respond appropriately with biological adaptations. Mechanical stimulation acts through these avenues to maintain homeostasis or create disturbances that can lead to disease. Mechanotransduction is well-known for influencing the attached extracellular matrix through production and activation of ECM proteins and matrix metalloproteinases (MMPs). (1) The events are crucial to maintain a homeostatic distribution of forces and ensure proper matrix turnover. Mechanical stimulation is thus an essential feature for the remodeling and production of the extracellular matrix of the PDL. In periodontal disease these processes are over stimulated by bacterial imbalance, interaction with the ECM and pathogenic activation of inflammatory mediators.

In the oral microbiome there has been over 700 identified species varying from symbiotic, commensal, and pathogenic relationships with the host. (2) For this reason, oral diseases like periodontitis and cancer manifest from a collective effort of dysbiosis from many different pathogens. Classically, research has approached this problem by identifying the most common recurrent bacteria and their relative abundance compared to healthy periodontal tissue load. From this idea, Socransky et al was able to characterize pathogens versus commensals based on abundance and severity of disease. Bacteria were characterized from low to high risk with the highest being defined as the “Red-complex”. (3) While these categories have been helpful to focus research, all pathogens do not have the same role in initiating disease. Additionally, all patients with periodontally compromised tissues do not have the same ratio of bacterial load. Therefore,

it is important to understand roles for each bacterium to properly address the gathered effect of disease when unbalanced.

Given the involvement of these pathogens to cause excessive extracellular matrix disorientation and dysfunction, we see a clear path from dysbiosis to ECM matrix induced turnover. Several studies have confirmed the relationship between PD and an increase of production of matrix proteins (4, 5) It is hypothesized that this is due to an increase of their degradation through pathogens directly or indirectly. ECM destruction produces degradation products that have been identified as biomarkers for PD. Such biomarkers have been traced back to bacterial pathogenic activity directly through their individual bacterial proteases or as a virulent factor activating the innate immune system. For example, Fibronectin catalysis producing 40-kDa, 120-kDa, and 68-kDa fragments are highly associated with severe periodontitis sites and have been identified as biomarkers for periodontal disease status. (6) These fragments have been shown to initiate anoikis mediated cellular death of periodontal ligament cells. (7, 8) *Porphyromonas gingivalis* and *Treponema denticola* have been implicated in vitro as pathogens that are responsible for producing these findings and influencing enhancement of MMPs to further catalyze these targets. (9, 10)

From these observations, the clinical field understands the need to address these pathogens in order to properly resolve periodontitis. According to the current trimeric model of treatment planning, periodontitis and bacterial load maintenance is approached by phase I and II periodontal therapy. (11) Phase I treatment focuses on diminishing bacterial infection by mechanical debridement of calculus and broad-spectrum antibiotics such as azithromycin, doxycycline, tetracycline, moxifloxacin, and amoxicillin/metronidazole. Phase II periodontics focuses on eliminating diseased tissues by increasing access for debridement of the tissues through creating flaps for gingival surgery (gingivectomy and gingivoplasty). Phase II approaches typically are effective by removing tissue instead and treating remaining infected tissue by neutralizing pro-inflammatory process and reduce abundance of pathogens. Phase II periodontal treatment at times accompanies adjunctive drug therapy including anti-tissue

destructive (sub-antimicrobial dose doxycycline) along with longer lasting techniques to deliver antibiotics such as Atridox (doxycycline), PerioChip (chlorohexidine glutamate), and Arrestin (minocycline). At times, a combination antibiotic therapy of amoxicillin and metronidazole will be used as an adjunctive therapy for phase II therapy because of its broad-spectrum antibacterial effects. (12) High dosage of this combination has been shown to reduce pocket depth and clinical attachment loss in aggressive periodontitis after 3 months for short term effects, but unless bacteria intervention is effective and remains in check, these parameters will relapse. (13, 14, 15) Meanwhile, the fact still remains that consistent intracellular infection can reduce susceptibility to antimicrobials leading to higher incidences of treatment failure (16). Together these findings suggest more studies are needed to address the compounding evidence that periodontal disease pathogens are skillfully able to persist intracellularly and extracellularly within periodontium tissues. Secondly, these findings suggest that in order to properly address microbial load of these pathogens, therapies are needed to target these areas of infection with concentrations that can efficaciously remove the load, without creating resistant strains due to prolonged use. (17) Furthermore, more evidence is needed to suggest or deny that infected tissues can be saved after being infected if both intracellular and extracellular are diminished.

The current antibiotics used were developed and prescribed to address extracellular bacteria. Limitations to eliminating bacteria is having appropriate drug concentration in diseased sites. Metronidazole is effective against extracellular anaerobes but does not effectively penetrate host tissue to target intracellular pathogens. (18) Clindamycin is effective in killing extracel*lular P. gingivalis* and *P. intermedia.(*18, 19) Yet, little evidence shows effective clearance of intracellular periodontal pathogens within periodontal tissues by the aforementioned antibiotics prescribed for phase I and II periodontitis treatment. Studies concomitantly focus shifted to developing antibiotics triggering inflammatory cells to internalize pathogens via endocytosis and killing bacteria in lysosomes. For example, clindamycin has shown to improve phagocytosis of *A. actinomycetemcomitans (AA)* and *P. gingivalis* (*Pg*) by polymorphic neutrophils (PMN) of healthy patients in vitro. Azithromycin also enhances Neutrophil phagocytosis of *AA*

in vitro. (20) Another avenue of research is addressing inflammatory-mediated periodontal destruction through the use of the anti-inflammatory class tetracyclines. Actions of tetracyclines and their derivatives lack antimicrobial action and include interactions with MMPs, tissue inhibitors of MMPs, growth factors and cytokines. (21, 22, 23)

Another challenge in identifying a promising antibiotic is the difference in bacterial susceptibility between species. One study showed doxycycline and clindamycin at a concentration 10-fold MIC had no effect in killing *Pg*, while metronidazole was effective. Within the same experimental conditions, intracellular *AA* was effectively killed doxycycline and moxifloxacin. (18). Additionally, Beikler et al. summed this idea with his study in 2004. From the subgingival plaque of 774 patients, nine major pathogen complexes (PCs) were found in 73.4% of all 774 patients, whereas 38 minor PCs were distributed in 26.6% of all patients. (24) The results of the study indicate that there are at least 46 different combinations of the assessed periodontal pathogens in subjects with periodontitis, and at least 10 different antibiotic regimens might be required to specifically target the various pathogen complexes. (24)

When considering of all of these factors, a reasonable conclusion is that the complete removal of bacteria by the use of antibiotics alone seems to be impossible when taking into account MIC values needed to kill pathogens (at least 10x the MIC) and the selective targeting of bacteria with particular antimicrobial agents. (18) Additionally, with only 50% of the known 700 species in the oral microbiome identified, the following questions remain: Do we have the knowledge to summarily conclude all contributions of critical pathogens? Can antimicrobial agents be effective inside and outside periodontal tissues? Is there an anti-microbial agent that focuses on “negative” pathogens instead of the “beneficial” commensal bacteria known to promote health? Nisin, a bacteriocin produced by the commensal bacterium Lactococcus lactis, has been identified as a promising antimicrobial agent that may shed insight on the previous questions.

Nisin was approved for human use as a food preservative by the world health organization (WHO) and by the food and drug administration (FDA) (25, 26). To date, nisin has been used to inhibit oral, head,

neck, and non-small cell lung cancer tumorigenesis and aggressivity (27, 28, 29, 30). Nisin has been used as a topical application and mouthwash to treat oral biofilm dysbiosis and gingivitis in dogs. (31, 32) The Kapila lab has been a pioneer for nisin in the treatment of oral dysbiotic initiated diseases like head and neck squamous cell carcinoma and periodontal disease. Some of our most promising findings show nisin is an effective anti-microbial with the potential to disrupt oral biofilm formation in vitro and in vivo, and key periodontopathic bacteria at low concentrations without eliminating commensal aerobic organisms (33, 34, 35). Kapila lab has demonstrated within polymicrobial infection mouse models, that Nisin probiotic not only inhibits bacterial, but can prevent inflammatory tissue degradation, promote reparative proliferation of periodontal tissues, and influence a healthy microbiome. (29, 35, 36) Various publications have reported numerous benefits of nisin enhancing the innate immune system (26). Finally, as a Nisin is both a cationic and amphiphilic peptide, nisin has great potential to interact with host cell membranes and distribute to various intracellular compartments (37). Notwithstanding, nisin's effect has only been observed as an extracellular agent, but it is unknown if nisin is effective in inhibiting growth of intracellular periodontopathic pathogens. No publication to date has shown nisin targeting intracellular pathogens. Therefore, nisin has hopeful capability to inhibit periodontopathogen bacteria inside and outside tissues, diminish immune response to dysbiotic biofilms, and potentially reset infected periodontium tissues to healthy status. However, studies are needed to properly establish these truths.

These findings support nisin's potential as a therapeutic for periodontitis. Yet, there are still limitations. As a naked bacteriocin, nisin is sensitive to proteases, such as Proteinase K and pepsin, thus creating possibilities for enzyme inhibition and resistance mechanisms (38, 39). Enzymes able to specifically inhibit Nisin, such as Nisinase and the Nisin Resistance Protein, have been reported in literature (40, 41). Therefore, alternatives to overcome this shortcoming are highly desirable. Nanoparticles have been identified as a novel direction to fight resistant strains of bacteria, escape enzyme degradation, and delivering antimicrobial agents to desired tissue sites. (42, 43) This dissertation investigated overcoming

these obstacles with nisin through packaging inside the nanoparticle drug delivery system, solid-lipid nanoparticles (SLN).

Nanoparticles have been used to target pathogens with various periodontal disease antibiotic agents. Chitosan-nanoparticles containing metronidazole, minocycline and doxycycline has been used to determine ease for intracellular trafficking and anti-inflammatory effects to potentially treat periodontal disease. (44, 45, 46) Additionally, PDA functionalized mesoporous silica nanoparticles, PLGA nanospheres, and even Au nanoparticles have successfully been sued to deliver antimicrobial agents such as minocylince (47), doxycycline and tetracycline (48, 49) and azithromycin and clarithromycin (Emmanuel R., 2017) respectively. Notably, another promising nanoparticle SLN, has been previously used to deliver metronidazole and N-phenacylthiazolium bromide through the buccal mucosa. More broadly, SLN has been effective to target various organs within the body including, skin, kidney, brain, and the oral cavity to treat oral dysbiosis. (32, 51, 52, 53) The Kapila lab has reported numerous benefits of SLN-Nisin complex including increasing the efficacy and potency for bacterial growth inhibition, increasing biofilm formation disruption, and enhancing anti-cancer activity when compared to nisin alone. (34, 54, 55) We hypothesize that nisin may also be effective in targeting pathogens inside and outside of periodontal tissues without harming commensal bacteria or host periodontia. It is also of interest to investigate if SLN-Nisin can also reverse any adverse effects from *T. denticola* challenging.

In conclusion, the purpose of this thesis is to investigate the microbial influence on the periodontal ligament and surrounding extracellular matrix. We specifically focused on the “red-complex” oral periodontopathogen, *T. denticola*. Through our findings we show molecular evidence for a mechanism of *Td*-mediated adverse cytopathic effects leading to characteristics of severe periodontitis; and evidence for nisin as a potential therapeutic for reducing or reversing *Td*-mediated periodontopathic processes in vitro.

REFERENCES

1. Bildyug, N. (2016). "Matrix metalloproteinases: an emerging role in regulation of actin microfilament system." Biomol Concepts **7**(5-6): 321-329.
2. Aas J.A., Paster B.J., Stokes L.N., Olsen I., and Dewhirst F.E. (2005). Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol*; 43: 5721–5732 (PMC1287824)
3. Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., & Kent, R. L. (1998). Microbial complexes in subgingival plaque. Journal of clinical periodontology, 25(2), 134-144. (PMID:9495612)
4. Ana P., Dimitrije M., Draginja K., Ivan M., Mariola S. The markers of systemic inflammation in patients with chronic periodontitis: Leukocytes, C-reactive protein, and fibrinogen. *J. Prevent. Med.* 2013;1(3):43–49.
5. Al-Isa, M., Alotibi, M., Alhashemi, H., Althobiani, F., Atia, A., & Baz, S. (2019). Effect of non-surgical periodontal therapy on the fibrinogen levels in chronic periodontitis patients. *The Saudi dental journal*, *31*(2), 188–193. https://doi.org/10.1016/j.sdentj.2018.12.001
6. Huynh, Q. N., Wang, S., Tafolla, E., Gansky, S. A., Kapila, S., Armitage, G. C., & Kapila, Y. L. (2002). Specific fibronectin fragments as markers of periodontal disease status. *Journal of periodontology*, *73*(10), 1101–1110. https://doi.org/10.1902/jop.2002.73.10.1101
7. Dai, R., Iwama, A., Wang, S., & Kapila, Y. L. (2005). Disease-associated fibronectin matrix fragments trigger anoikis of human primary ligament cells: p53 and c-myc are suppressed. *Apoptosis : an international journal on programmed cell death*, *10*(3), 503–512. https://doi.org/10.1007/s10495-005-1880-5
8. Jee, S. W., Wang, S., & Kapila, Y. L. (2004). Specific pro-apoptotic fibronectin fragments modulate proteinase expression in periodontal ligament cells. *Journal of periodontology*, *75*(4), 523–530. https://doi.org/10.1902/jop.2004.75.4.523
9. Ghosh, A., Park, J. Y., Fenno, C., & Kapila, Y. L. (2008). *Porphyromonas gingivalis*, gamma interferon, and a proapoptotic fibronectin matrix form a synergistic trio that induces c-Jun N-

terminal kinase 1-mediated nitric oxide generation and cell death. *Infection and immunity*, *76*(12), 5514–5523. https://doi.org/10.1128/IAI.00625-08

10. Miao, D., Fenno, J. C., Timm, J. C., Joo, N. E., & Kapila, Y. L. (2011). The *Treponema denticola* chymotrypsin-like protease dentilisin induces matrix metalloproteinase-2-dependent fibronectin fragmentation in periodontal ligament cells. *Infection and immunity*, *79*(2), 806–811. https://doi.org/10.1128/IAI.01001-10
11. Azouni, K. G., & Tarakji, B. (2014). The trimeric model: a new model of periodontal treatment planning. *Journal of clinical and diagnostic research : JCDR*, *8*(7), ZE17–ZE20. https://doi.org/10.7860/JCDR/2014/8458.4623
12. Sgolastra, F., Petrucci, A., Gatto, R., & Monaco, A. (2012). Effectiveness of systemic amoxicillin/metronidazole as an adjunctive therapy to full-mouth scaling and root planing in the treatment of aggressive periodontitis: a systematic review and meta-analysis. *Journal of periodontology*, *83*(6), 731–743. https://doi.org/10.1902/jop.2011.110432
13. Zhao, H., Hu, J., & Zhao, L. (2021). The effect of drug dose and duration of adjuvant Amoxicillin-plus-Metronidazole to full-mouth scaling and root planing in periodontitis: a systematic review and meta-analysis. *Clinical oral investigations*, *25*(10), 5671–5685. https://doi.org/10.1007/s00784-021-03869-w
14. Karrabi, M., & Baghani, Z. (2022). Amoxicillin/Metronidazole Dose Impact as an Adjunctive Therapy for Stage II - III Grade C Periodontitis (Aggressive Periodontitis) at 3- And 6-Month Follow-Ups: a Systematic Review and Meta-Analysis. *Journal of oral & maxillofacial research*, *13*(1), e2. https://doi.org/10.5037/jomr.2022.13102
15. Yek, E. C., Cintan, S., Topcuoglu, N., Kulekci, G., Issever, H., & Kantarci, A. (2010). Efficacy of amoxicillin and metronidazole combination for the management of generalized aggressive periodontitis. *Journal of periodontology*, *81*(7), 964–974. https://doi.org/10.1902/jop.2010.090522
16. Kanungo S. Dutta S. Sur D. (2008) Epidemiology of typhoid and paratyphoid fever in India. J Infect Dev Ctries2: 454–460.

17. Haffajee, A. D., Patel, M., & Socransky, S. S. (2008). Microbiological changes associated with four different periodontal therapies for the treatment of chronic periodontitis. *Oral microbiology and immunology*, *23*(2), 148–157. https://doi.org/10.1111/j.1399-302X.2007.00403.x
18. Eick, S., & Pfister, W. (2004). Efficacy of antibiotics against periodontopathogenic bacteria within epithelial cells: an in vitro study. *Journal of periodontology*, *75*(10), 1327–1334. https://doi.org/10.1902/jop.2004.75.10.1327
19. de Fijter, C. W., Verbrugh, H. A., Heezius, H. C., van der Meulen, J., Oe, P. L., Donker, A. J., & Verhoef, J. (1990). Effect of clindamycin on the intracellular bactericidal capacity of human peritoneal macrophages. *The Journal of antimicrobial chemotherapy*, *26*(4), 525–532. https://doi.org/10.1093/jac/26.4.525
20. Lai, P. C., Schibler, M. R., & Walters, J. D. (2015). Azithromycin enhances phagocytic killing of Aggregatibacter actinomycetemcomitans Y4 by human neutrophils. *Journal of periodontology*, *86*(1), 155–161.
21. Agnihotri, R., & Gaur, S. (2012). Chemically modified tetracyclines: Novel therapeutic agents in the management of chronic periodontitis. *Indian journal of pharmacology*, *44*(2), 161–167. https://doi.org/10.4103/0253-7613.93841
22. Tilakaratne, A., & Soory, M. (2014). Anti-inflammatory Actions of Adjunctive Tetracyclines and Other Agents in Periodontitis and Associated Comorbidities. *The open dentistry journal*, *8*, 109–124. https://doi.org/10.2174/1874210601408010109
23. Oringer, R. J., & Research, Science, and Therapy Committee of the American Academy of Periodontology (2002). Modulation of the host response in periodontal therapy. *Journal of periodontology*, *73*(4), 460–470. https://doi.org/10.1902/jop.2002.73.4.460
24. Beikler, T., Prior, K., Ehmke, B., & Flemmig, T. F. (2004). Specific antibiotics in the treatment of periodontitis--a proposed strategy. *Journal of periodontology*, *75*(1), 169–175. https://doi.org/10.1902/jop.2004.75.1.169

25. De Arauz, L.J., Jozala, A.F., Mazzola, P.G. and Penna, T.C.V. (2009) Nisin biotechnological production and application: a review. Trends Food Sci Technol 20, 146–154.
26. Cotter, P.D., Hill, C. and Ross, R.P. (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3, 777–788.
27. Joo, N. E., Ritchie, K., Kamarajan, P., Miao, D., & Kapila, Y. L. (2012). Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis via CHAC1. *Cancer medicine*, *1*(3), 295–305. https://doi.org/10.1002/cam4.35
28. Kamarajan, P., Hayami, T., Matte, B., Liu, Y., Danciu, T., Ramamoorthy, A., Worden, F., Kapila, S., & Kapila, Y. (2015). Nisin ZP, a Bacteriocin and Food Preservative, Inhibits Head and Neck Cancer Tumorigenesis and Prolongs Survival. *PloS one*, *10*(7), e0131008. https://doi.org/10.1371/journal.pone.0131008
29. Kamarajan, P., Ateia, I., Shin, J. M., Fenno, J. C., Le, C., Zhan, L., Chang, A., Darveau, R., & Kapila, Y. L. (2020). Periodontal pathogens promote cancer aggressivity via TLR/MyD88 triggered activation of Integrin/FAK signaling that is therapeutically reversible by a probiotic bacteriocin. *PLoS pathogens*, *16*(10), e1008881. https://doi.org/10.1371/journal.ppat.1008881
30. Patil, S. M., & Kunda, N. K. (2022). Nisin ZP, an Antimicrobial Peptide, Induces Cell Death and Inhibits Non-Small Cell Lung Cancer (NSCLC) Progression in vitro in 2D and 3D Cell Culture. *Pharmaceutical research*, *39*(11), 2859–2870. https://doi.org/10.1007/s11095-022-03220-2
31. Howell, T.H., Fiorellini, J.P., Blackburn, P., Projan, S.J., Harpe, J. and Williams, R.C. (1993) The effect of a mouthrinse based on nisin, a bacteriocin, on developing plaque and gingivitis in beagle dogs. J Clin Periodontol 20, 335–339.
32. Cunha, E., Valente, S., Nascimento, M., Pereira, M., Tavares, L., Dias, R., & Oliveira, M. (2021). Influence of the dental topical application of a nisin-biogel in the oral microbiome of dogs: a pilot study. *PeerJ*, *9*, e11626. https://doi.org/10.7717/peerj.11626

33. Shin, J.M., Ateia, I., Paulus, J.R., Liu, H., Fenno, J.C., Rickard, A.H. and Kapila, Y.L. (2015) Antimicrobial nisin acts against saliva derived multi-species biofilms without cytotoxicity to human oral cells. Front Microbiol 6, 617.
34. Radaic, A., Ye, C., Parks, B., Gao, L., Kuraji, R., Malone, E., Kamarajan, P., Zhan, L., & Kapila, Y. L. (2020). Modulation of pathogenic oral biofilms towards health with nisin probiotic. *Journal of oral microbiology*, *12*(1), 1809302. https://doi.org/10.1080/20002297.2020.1809302
35. Gao, L., Kuraji, R., Zhang, M. J., Martinez, A., Radaic, A., Kamarajan, P., Le, C., Zhan, L., Ye, C., Rangé, H., Sailani, M. R., & Kapila, Y. L. (2022). Nisin probiotic prevents inflammatory bone loss while promoting reparative proliferation and a healthy microbiome. *NPJ biofilms and microbiomes*, *8*(1), 45. https://doi.org/10.1038/s41522-022-00307-x
36. Nguyen, T., Brody, H., Lin, G. H., Rangé, H., Kuraji, R., Ye, C., Kamarajan, P., Radaic, A., Gao, L., & Kapila, Y. (2020). Probiotics, including nisin-based probiotics, improve clinical and microbial outcomes relevant to oral and systemic diseases. *Periodontology 2000*, *82*(1), 173–185. https://doi.org/10.1111/prd.12324
37. Vater, M., Möckl, L., Gormanns, V., Schultz Fademrecht, C., Mallmann, A. M., Ziegart-Sadowska, K., Zaba, M., Frevert, M. L., Bräuchle, C., Holsboer, F., Rein, T., Schmidt, U., & Kirmeier, T. (2017). New insights into the intracellular distribution pattern of cationic amphiphilic drugs. *Scientific reports*, *7*, 44277. https://doi.org/10.1038/srep44277
38. Ansari, A., Zohra, R. R., Tarar, O. M., Ul Qader, S. A. & Aman, A. Screening, purification and characterization of thermostable, protease resistant Bacteriocin active against methicillin resistant Staphylococcus aureus (MRSA). *BMC Microbiol.* **18,** (2018).
39. Yang, S.-C., Lin, C.-H., Sung, C. T. & Fang, J.-Y. Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Front. Microbiol.* **5,** 241 (2014).
40. Jarvis, B. & Farr, J. Partial purification, specificity and mechanism of action of the nisin-inactivating enzyme from Bacillus cereus. *Biochim Biophys Acta* **227,** 232–240 (1971).

41. Kramer, N. E., Van Hijum, S., Knol, J., Kok, J. & Kuipers, O. P. Transcriptome analysis reveals mechanisms by which Lactococcus lactis acquires nisin resistance. *Antimicrob. Agents Chemother.* **50,** 1753–1761 (2006).
42. Baptista, P. V., McCusker, M. P., Carvalho, A., Ferreira, D. A., Mohan, N. M., Martins, M., & Fernandes, A. R. (2018). Nano-Strategies to Fight Multidrug Resistant Bacteria-"A Battle of the Titans". *Frontiers in microbiology*, *9*, 1441. https://doi.org/10.3389/fmicb.2018.01441
43. Basudan A. M. (2022). Nanoparticle based periodontal drug delivery - A review on current trends and future perspectives. *The Saudi dental journal*, *34*(8), 669–680. https://doi.org/10.1016/j.sdentj.2022.09.006
44. Martin, V., Ribeiro, I., Alves, M. M., Gonçalves, L., Almeida, A. J., Grenho, L., Fernandes, M. H., Santos, C. F., Gomes, P. S., & Bettencourt, A. F. (2019). Understanding intracellular trafficking and anti-inflammatory effects of minocycline chitosan-nanoparticles in human gingival fibroblasts for periodontal disease treatment. *International journal of pharmaceutics*, *572*, 118821. https://doi.org/10.1016/j.ijpharm.2019.118821
45. Mahmoud M.M., Samy W.M. Enhanced Periodontal Regeneration by Novel Single Application Sustained Release Nano-Structured Doxycycline Films. *Curr. Drug Deliv.* 2016;13(6):899–908.
46. Lee B.S., Lee C.C., et al. Controlled-release of tetracycline and lovastatin by poly(D, L-lactide-co-glycolide acid)-chitosan nanoparticles enhances periodontal regeneration in dogs. *Int. J. Nanomed.* 2016;11:285–297.
47. Bai B., Gu C., et al. Polydopamine functionalized mesoporous silica as ROS-sensitive drug delivery vehicles for periodontitis treatment by modulating macrophage polarization. *Nano Res.* 2021;14(12):4577–4583.
48. Lecio G., Ribeiro F.V., et al. Novel 20% doxycycline-loaded PLGA nanospheres as adjunctive therapy in chronic periodontitis in type-2 diabetics: randomized clinical, immune and microbiological trial. *Clin. Oral Investig.* 2020;24(3):1269–1279.

49. Ranjbar-Mohammadi M., Zamani M., et al. Electrospinning of PLGA/gum tragacanth nanofibers containing tetracycline hydrochloride for periodontal regeneration. *Mater. Sci. Eng., C.* 2016;58:521–531.
50. Emmanuel, R., Saravanan, M., Ovais, M., Padmavathy, S., Shinwari, Z. K., & Prakash, P. (2017). Antimicrobial efficacy of drug blended biosynthesized colloidal gold nanoparticles from Justicia glauca against oral pathogens: A nanoantibiotic approach. *Microbial pathogenesis*, *113*, 295–302. https://doi.org/10.1016/j.micpath.2017.10.055
51. Katari, O., & Jain, S. (2021). Solid lipid nanoparticles and nanostructured lipid carrier-based nanotherapeutics for the treatment of psoriasis. *Expert opinion on drug delivery*, *18*(12), 1857–1872. https://doi.org/10.1080/17425247.2021.2011857
52. Radaic, A. & de Jesus, M. B. Solid Lipid Nanoparticles release DNA upon endosomal acidification in Human Embryonic Kidney Cells. *Nanotechnology* **29,** 1–10 (2018).
53. Shankar, R., Joshi, M., & Pathak, K. (2018). Lipid Nanoparticles: A Novel Approach for Brain Targeting. *Pharmaceutical nanotechnology*, *6*(2), 81–93. https://doi.org/10.2174/2211738506666180611100416
54. Radaic, A.; de Jesus, M.B.; Kapila, Y.L. Bacterial Anti-Microbial Peptides and Nano-Sized Drug Delivery Systems: The State of the Art toward Improved Bacteriocins. *J. Controlled Release* **2020**, *321*, 100–118, doi:10.1016/j.jconrel.2020.02.001.
55. Radaic, A.; Malone, E.; Kamarajan, P.; Kapila, Y.L. Solid Lipid Nanoparticles Loaded with Nisin (SLN-Nisin) Are More Effective Than Free Nisin as Antimicrobial, Antibiofilm, and Anticancer Agents. *J. Biomed. Nanotechnol.* **2022**, *18*, 1227–1235, doi:10.1166/jbn.2022.3314.

Chapter 1.1: ABSTRACT

Periodontal disease is characterized by destruction of the hard and soft tissues that comprise the periodontium. Pathogenic microbes are a catalyst for periodontal disease pathogenesis since the development of a dysbiosis of the microbiome enhances a persistent immune response. Oral spirochete, *Treponema denticola* (*T. denticola or Td*), is highly associated with periodontal disease (1, 2). We've demonstrated that a *Td* outer membrane protein complex called dentilisin, contributes to the chronic activation of pro-MMP-2 in periodontal ligament (PDL) cells (3, 4) Dentilisin exposure also triggers increased expression levels of activators and effectors of active MMP-2 in PDL cells. Despite these advances, there is no mechanism known for dentilisin-induced MMP-2 activation or PDL cytopathic behaviors leading to disease, or potential treatment. One possible therapeutic to combat these interactions is nisin. Nisin is a naturally occurring bacteriocin, widely used food preservative, and probiotic agent naturally made by bacterial species similar to those in the gut and oral cavity. Our lab demonstrated that nisin is an effective anti-microbial against key periodontopathic bacteria at low concentrations, including *Td,* with the potential to disrupt oral biofilm formation in vitro (5). Furthermore, nisin at low concentrations is also more effective against certain oral pathogenic versus commensal bacteria, making it a potentially useful and selective agent for oral applications. Proper clearance of *Td* may provide a more symbiotic microbiome and "reset" homeostatic conditions needed for repair, remodeling, or regeneration of the periodontium. Thus, the main hypotheses of this proposal are that *T. denticola* interactions with PDL cells mediate adverse effects on homeostasis and cellular functions leading to a compromised cellular phenotype. Additionally, nisin is a potential therapeutic for abrogating effects of MMP-2. This hypothesis will be tested in the following specific aims: *1) Determine the mechanism of dentilisin-induced activation of pro- MMP-2 in PDL cells; 2) Characterize the effects of T. denticola (Td) challenge on PDL cell processes such as viability, migration, and contractility.*

Chapter 1.2: INTRODUCTION

Periodontal disease (PD) is characterized by destruction of the hard and soft tissues that comprise the periodontium. This destruction translates to a degradation of the extracellular matrices (ECM), which is mediated by host-derived proteases, such as matrix metalloproteinases (MMPs) and bacterial proteases. Pathogenic microbes are a catalyst for periodontal disease pathogenesis, since they perpetuate a microbial dysbiosis that triggers a chronic host immune response. In periodontal disease, the “red complex” group of pathogenic bacteria have been strongly implicated in the pathogenesis of the disease. One member of the “red complex”, the oral spirochete, *Trepenoma denticola* (*T. denticola*), is highly associated with periodontal disease (5). Bacteremias of oral bacteria, including red complex bacteria, such as *T. denticola*, gain access to various body tissues, and thereby induce or aggravate various systemic diseases (6, 7, 8, 9) Additionally, there is a positive correlation between the proportions of subgingival spirochetes and susceptibility of human subjects to periodontal deterioration (10). Tissue destruction and chronic inflammation are hallmarks of periodontal disease, and other associated systemic diseases. For example, human data and animal models of atherosclerosis provide evidence that periodontal pathogens enhance the systemic effects of the disease through inflammatory mechanisms (11,12). Although bacterial endotoxins like LPS are thought to trigger these immune responses, some oral pathogenic bacteria, like *T. denticola* do not contain LPS. Thus, other bacterial virulence factors also contribute to disease pathogenesis.

Publications in the Kapila Laboratory, among other studies, show a functional role for the *T. denticola* outer membrane component, dentilisin, in contributing to periodontal disease mechanisms. Specifically, dentilisin enhances MMP-2 activity and specific fibronectin cleavage; similar to the fibronectin fragments that are known as disease biomarkers for periodontitis patients (3, 4, 13). Other studies also show that *T. denticola* is mechanistically involved in inducing the phenotypic characteristics of periodontitis, and suggest that its outer membrane component, dentilisin is involved. (14) Still, little is known about the mechanisms by which these *T. denticola* components mediate these processes. Also, the general focus

of most in vitro studies have involved *T. denticola* effects on immune cells and gingival epithelium, but little is known about the microbial effects of *T. denticola* on periodontal ligament (PDL) cells.

PDL cells are known to function in maintaining bone, periodontal tissues, the extracellular matrix, forming new connective tissue attachments all performed largely through MMPs (14,15). MMPs are involved in various biological processes such as cell proliferation, mechanotransduction, migration and wound healing. (17, 18) All of these functions are perturbed in periodontal disease progression. (19,20) Therefore, understanding the microbial-host interactions that alter the PDL cell phenotype is essential to understating the complexities of periodontal disease pathogenesis. Despite these advances, there is no known mechanism for dentilisin- mediated MMP-2 activation or PDL cytopathic behaviors leading to disease.

A better understanding of host-bacterial interactions influencing PDL cell homeostasis is needed to help develop clinical therapeutics to address chronic PD. Thus, the main hypothesis of this chapter is that *T. denticola* interactions MMP-2 and PDL cells, has adverse effects on normal homeostasis and cellular functions leading to a severely compromised cellular phenotype. We hypothesize that *T. denticola* will have a significant effect on PDL cell cytopathic functions, MMPs direct activation, and indirectly through stimulating molecular signaling in PDL cells.

Chapter 1.3: RESULTS

1.3.1 Dentilisin may directly activate MMP-2

Several activating enzymes for pro-MMP-2 have been proposed, including metalloproteases and serine proteases (8). This is corroborated by our findings, and in addition, our data further suggest that the serine protease, dentilisin also activates MMP-2 in PDL cell cultures (4, 5, Fig. 2). However, the mechanism by which this activation occurs is unknown. Dentilisin and pro-MMP-2 have similar sequence interactions predicted by BLASTp analysis (Supplemental Table 1.7.1). These suggested interactions are located on the C-terminus of MMP-2, where the homeopexin domain is located, specifically called the pfam00045. The homeopexin domain was coined the "helping hand" to collagenase-13 (MMP-13), due to its proposed function of conferring characteristic binding and cleavage specificity in collagenases for triple-helical substrates. Equivalent domain structures were found in the C-terminal end of fibroblast collagenase 1 (MMP-1) and gelatinase A (MMP-2) (25). The other suggested Homepexin-like repeat (smart00120), located in the same region of the MMP-2 sequence, has a similar domain sequence as pfam00045, and is suggested to bind tissue inhibitor of metalloproteinases (TIMPs), vitronectin, and some MMP family members (26, 27, 28, 29). Both domains are found in MMP-2 according to this NCBI search (23, 24). Based on these results, I formulated a hypothesize that dentilisin can interact with pro-MMP-2 through this domain alone, or in a complex with MT1-MMP, to activate proMMP-2.

Per manufacturer's instructions, I used EnZcheck Gelatinase/Collagenase Activity Assay Kit (ThermoFischer e12065) to perform gelatin zymography within a 96 well plate. This system has been used to determine gelatinase activity of MMPs and inhibition of gelatinase degradation. (77) To test our hypothesis, I obtained purified dentilisin from Dr. JC Fenno our collaborator using his purified techniques (23) and purchase purified recombinant pro-MMP-2 from (R&D Systems, Minneapolis, MN;

Prospec, East Brunswick, NJ; Sino Biological, Beijing, China; EnzoLifeSciences; product number: ALX-200-419- C005). Sample groups were control (gelatin substrate and reaction buffer), purified pro-MMP-2 with gelatin substrate and reaction buffer, purified dentilisin with gelatin substrate and reaction buffer, or both MMP-2 and dentilisin with gelatin substrate and reaction buffer. Pierce's BCA protein quantification was used to determine initial concentration of each purified protein and to dilute to a final concentration of 1ug/ml with reaction buffer. Our results were inconclusive. We did see an increase of gelatin degradation at all timepoints post-incubation. We saw an increase in equal parts of dentilisin + MMP-2 compared to all groups. Another thought for the increase in degradation is a synergistic effect as opposed to an activation of MMP-2 by dentilisin. (Figure 1.3.1A) In order to rule out this presumption, I combined the totals for MMP-2 alone and Dentilisin alone to compare this total to the MMP-2 and Dentilisin sample. At the 2hrs, there is a significant increase in gelatinase degradation thus suggesting MMP-2 activation as opposed to an additive effect. (Figure 1.3.1B)

To finalize this question, MMP-2 and dentilisin incubation and western blotting for pro- and active-MMP-2 forms is necessary. Our studies using whole bacteria *T. denticola* reveals increased pro-MMP-2 production through enhanced transcription of MMP-2 and its effector/activator complex TIMP-2 and MMP-14, but not necessarily increase in the active form of MMP-2. (22, 5, 31, and 32) Furthermore, physiologically in diseased environments fibrinogen plays a role in inhibiting MMP-2, but not MMP-2. In periodontal compromised tissues, *T. denticola* is suggested to degrade fibrinogen leaving fragments recognized as a biomarker for severely infected tissues. Therefore while *T. denticola* may not activate MMP-2 directly, it does influence the abundance of the protein, its mediators for activation, and decreases the abundance of its extracellular matrix inhibitors. All factors are crucial in tipping the balance of healthy tissue toward a diseased environment.

1.3.2 *T. denticola* interaction with hPDL cells enhances viability and metabolism

The Calcein AM assay will be used to measure the effects of *T. denticola* and dentilisin on PDL cell proliferation. The Calcein AM Assay Kit was used to determine changes in cell number using a standard microplate absorbance reader. The Calcein AM kit was purchased (ThermoFisher Scientific; catalog number C1430) and used according to the manufacturer's protocol and as previously described 31). Human PDL cells were measured after 24 hrs of incubation with Wild-Type *T. denticola* and dentilisin inactive *T. denticola* mutant (*Td*-P7076) at different *T. denticola* MOIs (10, 50, 100, 250, and 500) were used. Calcein AM assay showed *T. denticola* interaction does not diminish viability of PDL cells. (Figure 1.3.2) *T. denticola* at an MOI of 10, 50, and 100 all showed a significant increase in metabolic activity, while *T. denticola* at an MOI of 500 showed a significant decrease signifying cytotoxicity. The trend is lessened with *T. denticola* inactive dentilisin mutant interaction with at MOI 10 enhancing metabolic activity significantly and MOI of 500 showing significant drop in activity. Our results show that *T. denticola* interaction trigger metabolic activity and dentilisin plays a significant role in this enhancement in human PDL cells. (30)

1.3.3 *T. denticola* enhances Migration/ Wound healing in hPDL cells

Human PDL cells are integral cells in maintain homeostatic physiologic conditions by initiating wound healing and regeneration of the periodontium. The cellular process of migration is needed to fill in tissue perturbations and gaps due to inflammatory response and disease. (34; 69) In the context of oral cancer, *T. denticola* has been shown to encourage migration through upregulation of integrins and FAK transcriptionally and at a protein level (33) Additionally, *T. denticola* supernatants cultured with periodontal ligament stem cells illicitated a migratory response. (37) Conversely, *T. denticola* and its Major surface protein (MSP) disrupts the function of epithelial cell wound healing and migration

(35) I hypothesize Wild-Type *Td* will also induce enhanced migration among human PDL cells. Migration assays were used to determine the effect of *T. denticola* on PDL cell migration. Migration assay protocols were those used in the Kapila Laboratory, which have been previously described (21). Migration results showed a 50% increase in wound healing/migration of *T. denticola* challenged hPDL cells compared to unchallenged cells. (Figure 1.3.3) Therefore, we can conclude that whole bacteria WT-*T. denticola interaction* enhances migration in Periodontal Ligament Cell.

1.3.4 *T. denticola* does not significantly decrease contractility in hPDL cells

Human PDL cells are constantly sensing the tensile forces of the tissue and extracellular matrices. (39) A function of this sensing is the ability to reorganize the cytoskeleton to contract and expand. (40) *T. denticola* has been shown to perturb the cytoskeleton in gingival fibroblasts by using its major surface protein to induce actin assembly in a PIP2-dependent manner. *T. denticola* has also been shown to interact with integrins alpha5B1 through its *Td*92 surface protein and collagen. (41) *Td* binding to Type I, IV, and V by its collagen binding proteins prevent fibroblast interaction with collagen through subcortical actin assembly and Beta1 integrin affinity. (42, 43, 44, 45) May be crucial in preventing the PDL from aligning collagen fibers necessary for tissue contractility. Therefore, I hypothesized that *T. denticola* could alter the contractile ability of PDL cells. Results from the collagen contractility assay demonstrated a small biologic effect on hPDL cells reducing contractile ability through interaction of Td at MOI of 50 compared to no challenge. (Figure 1.3.4) While, the experiments did not show expected results, live imaging of actin cytoskeleton contractility was visually impaired, resulting in cellular detachment.

Chapter 1.4: DISCUSSION

In summary, research findings implicate that periodontal pathogenesis acts heavily through the bacterial capacity to bind to ECM proteins. Binding complexes aid the pathogen to do the following: 1) cleave and degrade ECM proteins, 2) cleavage and degradation of host cell receptors through proteolytic activities; 3) host cell stimulation leading to overexpression of tissue destructive genes, pro-inflammatory signal transduction, and bacterial entry/invasion inside the cell. All heavily studied pathogens implicated in periodontal disease produce proteins to help accomplish these goals. The following are a few examples of these effector proteins from the red-complex classification:

Porphyromonas gingivalis (*Pg*) is the most studied periodontal pathogen among the red-complex category. *Pg* is known to form complexes with fimbriae or KgP to expose ECM protein receptors and maintain strong adherence to laminin, collagen, and fibronectin, which in turn leads to degradation of ECM proteins and integrins, respectively. (46, 47, 48) *Pg* contains effector proteins, gingipains which have proteolytic activities to degrade immune receptors, complement components, host surface proteins, and activates metalloproteases. (46) *PG* also uses gingipains to invade oral epithelial cells and degrade intracellular signaling proteins, like mammalian target of rapamycin (mTOR). (49)

The Gram-negative anaerobe *Tannerella forsythia* (Tf) is a member of the *Cytophaga-Bacteroides* and is implicated in the onset of periodontitis and progressive destructive lesions in periodontal disease. [50] Tf has binding capabilities to fibronectin and fibrinogen-binding proteins through Bacteroides surface protein A (BspA) and Tf1331. One of the most abundant effector proteins in periodontially- compromised patients is *bfor_1659*, a secreted dipeptidyl aminopeptidase IV (DPP IV). (51, 52) DPP IV functions as a serine protease which cleaves type I and III collagen, contributing to periodontal tissue destruction. [53, 54]. Tf also has a matrix metallopeptidase which is similar in structure and activity to mammal metalloproteinase protein named Karilysin (Kly18). (55; 56) Kly18 has the capability to degrade elastin,

fibrinogen and fibronectin. (55) To date, Kly18 is the only bacterial MMP to have been characterized biochemically. Along with the virulence effect of Tf ECM binding and degradation, Kly18 may be another contributor to the pathogenicity of periodontitis. (57)

The spirochete *Treponema denticola* is equipped with adhesins capable of binding to extracellular matrix proteins [58]. Dentilisin, major surface protein (MSP), and outer membrane–associated protein (OppA) are well-known effector proteins known to bind and/or cleave ECM proteins. The major surface (sheath) protein (Msp) is a porin-like protein found in the outer membrane of *T. denticola* is an important adhesin capable of binding to fibronectin, laminin, fibrinogen, and collagen I [58]. OppA binds laminin, fibronectin and plasminogen. [59. Dentilisin, a chymotrypsin-like protease, acts as a major virulence factor in this species that binds to and degrades several basement membrane components, including fibronectin, laminin, and collagen IV, facilitates bacterial migration, and activates MMP-2 indirectly. (36, 60, 4, 5) Finally, dentilisin, Msp, lipoteichoic acid, and periplasmic flagella of *Td* have all been demonstrated as pathogen-associated molecular patterns (PAMPs) that are recognized through toll-like receptor to stimulate the innate immune response. (32, 61, 62, 63)

In conjunction with these findings, our study supports dentilisin as an activator of MMP-2 directly. This is the first study to suggest direct activation of MMP-2 by dentilisin. Another publication demonstrates *Td* chymotrypsin like protein (dentilisin) was found to convert pro MMP-8 and -9 into their active forms and degrade the proteinase inhibitors TIMP-1, TIMP-2, and α-1-antichymotrypsin. (64) Indirectly, We can conclude that periodontopathogens *Td*, *Pg*, and *Tf* play a major role in activating MMPs and maintaining excessive ECM turnover through their bacterial protease activity.

Treponema denticola infection effects on periodontal ligament cellular functions have yet to be described. To date, regarding cellular viability and contractility, no publication has documented these cytopathic effects in human PDL cells. Therefore, our studies lay the foundation for *Td*-mediated increase in

viability/metabolism and decreased contractility. Largely focus has been on the *Td* effect on epithelial cells in general. *Wild-type T. denticola* can disrupt transepithelial resistance and cell contact junctions. (65; 36) Loss of these attachments are important in wound closure and migration. Clinically, apical migration is a key characteristic in periodontal disease and was seen in a *Pg* and *Td* mixed infection rat model. (66) While, cellular migration of epithelial cells was diminished, our studies show PDL cells reacted differently to *Td* challenge by enhancing migratory effect. (35) The increase in migration is reversed by cytochalasin D inhibitor suggesting the *Td* effect is driven by actin reorganization. In accordance with our study, *Td*, *Pg*, and *FN* enhance migration, invasion, and tumorigenesis in OSCC cells through TLR/MyD88 crosstalk. (33) Kapila lab has shown this same mechanism results in MMP-2, 11, 14, 17, and 28 transcriptional upregulation due to dentilisin interaction with hPDL cells. (32) Therefore, dentilisin may be the potential mediator for PDL cell migratory events.

1.5 MATERIALS AND METHODS

Human Subjects and IRB Approval

Institutional review board (IRB) approval for human subjects research was obtained via the University of California San Francisco institutional review board (# 16-20204; reference #227030).

Periodontal Ligament Cell Harvesting and Cell Culture

Human primary periodontal ligament (PDL) cells were grown as explants from periodontal ligament tissues that were harvested from extracted teeth as previously described (68). All cells were cultured in alpha minimal essential medium (MEM-α) supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin-streptomycin, and 1% amphotericin B (Gibco, USA). All cells were used between passages 3 and 7. Use of PDL cells for these studies was approved by the University of California San Francisco IRB. Two donor samples were pooled together per independent sample to eliminate variation for all experiments except shRNA experiments (only one donor per independent sample was used for these experiments).

Microbial Culturing of *T. denticola*

T. denticola ATCC 35405 (American Type Culture Collection; Manassas, VA) and *T. denticola* mutants (CF522, isogenic dentilisin-deficient strain) (67), MHE (isogenic Msp-deficient strain) (Fenno, Wong et al. 1997), were cultured at 37°C under anaerobic conditions in oral treponeme enrichment broth (OTEB; Anaerobe Systems, Morgan Hill, CA). Culture purity was monitored using Syto 9 bacterial DNA dye and visualized in a fluorescent microscope for spirochete morphology. *T. denticola* was washed with MEM-α cultured media, centrifuged, aspirated thrice, and finally resuspended in MEM-α to be used for experiments. Bacteria used for all experiments never exceeded 6 passages.

Purified Dentilisin

The dentilisin protease complex was purified from the detergent phase of the Triton X-114 extracts of *T. denticola* MHE by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a model 491 Prep Cell (Bio-Rad Laboratories, Richmond, CA) as described previously (23, 21). Dentilisin purity was assayed in silver stained SDS-PAGE (21). Purifed dentilisin migrated as a 100 kDa complex that upon heating resolves to its individual protein components: PrtP, PrcA1 and PrcA2, as documented previously (67)."

MOI Optimization

Multiplicity of infection (MOI) was optimized by performing a PDL cell viability assay with *T. denticola* at different MOIs (10, 50, and 100). This viability assay (Calcein AM; Thermo Fisher Scientific) was used according to manufacturer's instructions. Cytotoxicity was encountered at an MOI of 500 (data not shown). In previous publications (5, 22), a *T. denticola* MOI of 50 rendered a strong cellular response in PDL cells without inducing cytotoxicity. Based on these observations, an MOI of 50 was considered optimal for studying the cellular response of PDL cells to *T. denticola* treatment.

Treatment of PDL cells with *T. denticola*

PDL cells were plated in 4-well chamber slides (Invitrogen), 8-well chamber slides (Invitrogen), or 60mm culture plates (Falcon) overnight at a density of 3x104, 1.5x104 or 1.0x106 cells/well, respectively. Cells were then challenged with *T. denticola* at an optimized MOI of 50 for 2 h or left unchallenged as controls. Post-challenge, cells were washed three times with phosphate buffered saline (PBS, Gibco, USA) and incubated in MEM-α media (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% amphotericin B (Gibco, USA), and 1% penicillin-streptomycin for 24 h.

General Chemicals

All general chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise noted.

In situ Gel Zymography Assay

EnzChek™ Gelatinase/Collagenase Assay Kit (cat# E12055; Invitrogen), was used per manufacturer's instructions for assays for gelatinase activity in a 96-well plate format. Purified dentilisin was obtained from Dr. JC Fenno our collaborator using his purified techniques (13) and purchase purified recombinant pro-MMP-2 from (R&D Systems, Minneapolis, MN; Prospec, East Brunswick, NJ; Sino Biological, Beijing, China; EnzoLifeSciences; product number: ALX-200-419- C005). 80uL of 1X Reaction Buffer was added to each well. 20 uL of the 1.0 mg/mL DQ gelatin stock was used to obtain a 100ug/mL final subtrate concentration. Both MMP-2 and Dentilisin was Sample groups were control (gelatin substrate and reaction buffer), purified pro-MMP-2 with gelatin substrate and reaction buffer, purified dentilisin with gelatin substrate and reaction buffer, or both MMP-2 and dentilisin with gelatin substrate and reaction buffer. Pierce's BCA protein quantification was used according to manufacturer's instructions to determine initial concentration of 1.0mg/mL for each purified protein. Further dilution to a final concentration of 0.002 mg/ml with reaction buffer was achieved similar to previously described publication. (38). 100uL of final concentration of 1X reaction buffer (negative control), MMP-2 alone, or Dentilisin alone was added to respective grouped wells. 50uL of final concentration MMP-2 plus 50uL of final concentration dentilisin was added to its respective well group. Samples were read in a fluorescent microplate reader at 495nm absorption and 515nm emission. Samples were read at 0, 30, 60, 90, and 120min timepoints. Data represent mean ± SD from two independent experiments containing wells per sample. Data was compared using One-way ANOVA Tukey's Multiple comparisons test. ***= $p<0.001$; N.S.= not significant

Migration Methods

A confluent monolayer (approximately 40,000 cells) will be seeded by well in a 96-well plate. Cells will adhere and reconstitute overnight. Cells were unchallenged (control), challenged by *T. denticola* at 50 or 100 MOI or with an endocytosis inhibitor (Wortmannin, Cytochalasin D, and Methyl-Beta

Cyclodextrin) at MOI: 50 and 100) for 2 hours. Inhibitors inhibit macropinocytosis (Wort), phagocytosis (CytoD), and caveolin-mediated endocytosis (MBCD). A scratch wound was made by a micropipette to form an even wound, washed three times with PBS, and reconstituted with MEM-α media. Time course images were taken (0, 24, and 48hrs) under a light microscope. Images were opened in FIJI imaging software, borders were outlined, and area of the outline was taken at each timepoint and compared to control PDL cells. 3-A. Light microscopy images were taken at 0hr, 24hr, and 48hr time points. 3-B. Representatives quantitative measurements of the total migratory distance of cells from edges of the wound.

Viability Assay Methods

PDL cells were plated in 96 well plate (approximately 3x 10 cell per well) unchallenged (control) or challenged with *T. denticola* (10, 50, or 100 MOI) for 24 h and Calcein AM assays were performed according to manufacturer's instructions. Data represent mean ± SD from three independent experiments containing 10 wells per sample. Data was compared using One-way ANOVA Tukey's Multiple comparisons test. *= $p<0.05$; N.S.= not significant

Contractility Methods

PDL cells were challenged for 2hrs, wash 3x with PBS, trypsinized and spun down. Cells were counted and seeded to 6x10^6 cells per mL of media plus collagen gel mix. Final volume concentration is approximately 2.0mg/mL of collagen type I with pH adjusted to 7.5. Cells were seeded 1x10^6 cells per well in a 24 well plate. After 1hr incubation, 1mL of MEMα medium was added to each well. After another 3hrs of incubation, use a pipette tip to trace around the well to remove the collagen gel from the bottom of the plate. Photos were taken of the contracted collagen gel on a dissecting microscope for 0hr, 24hrs, and 44hrs time points. FUJI imaging software was used to calculate the change in gel circumference between conditions.

Chapter 1.6: FIGURES

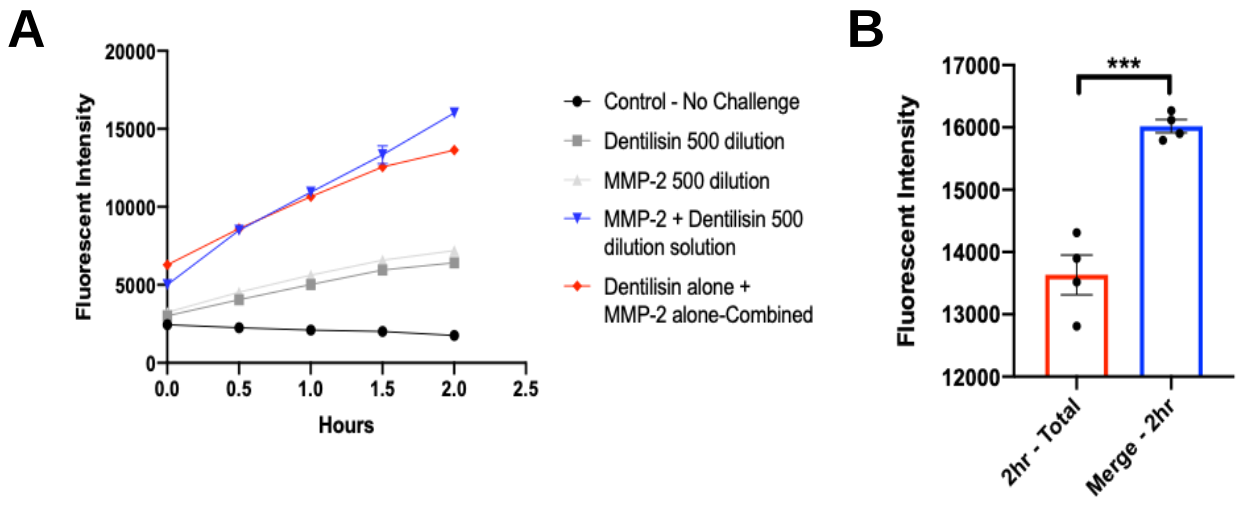


Figure 1.3.1 Direct activation of pro-MMP-2 by dentilisin by in situ gel zymography. Fluorescent intensity of each group was plotted at each interval (0, 0.5, 1, 1.5, 2hr). Sample groups were positive control (Black), purified pro-MMP-2 alone (Light Grey), purified dentilisin (Dark Drey), or both MMP-2 and dentilisin (Blue) (Figure 1A). Combined totals for MMP-2 alone and Dentilisin alone were also plotted and compared to rule out additive effect. (Figure 1B) Plot represents average of two experiments with 6 separate samples in each group per experiment. . Data was compared using One-way ANOVA and Tukey's Multiple comparisons test. ***= $p<0.001$; N.S.= not significant

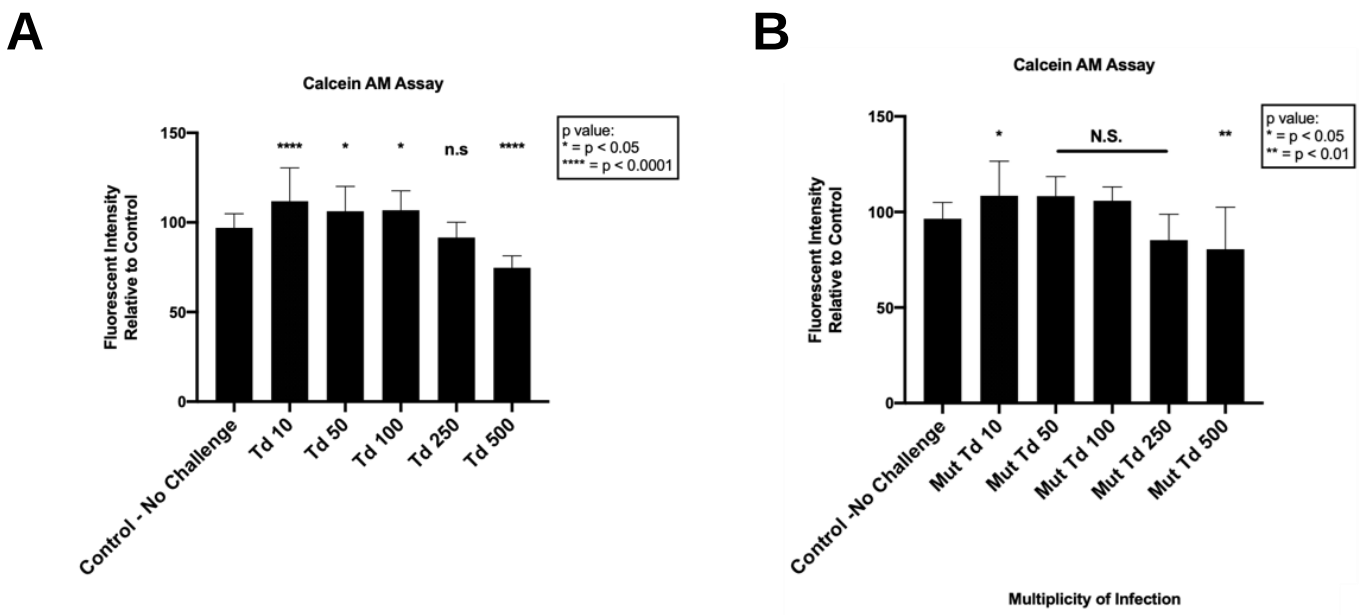


Figure 1.3.2 Wild-Type *Treponema denticola* alters viability/metabolism activity in hPDL cells. PDL cells were plated in 96 well plate (approximately 3x 10 cell per well) unchallenged (control) or challenged with *T. denticola* (10, 50, or 100 MOI) for 24 h and Calcein AM assays were performed according to manufacturer's instructions. Data represent mean ± SD from three independent experiments containing 10 wells per sample. Data was compared using One-way ANOVA Tukey's Multiple comparisons test. *= $p<0.05$; N.S.= not significant

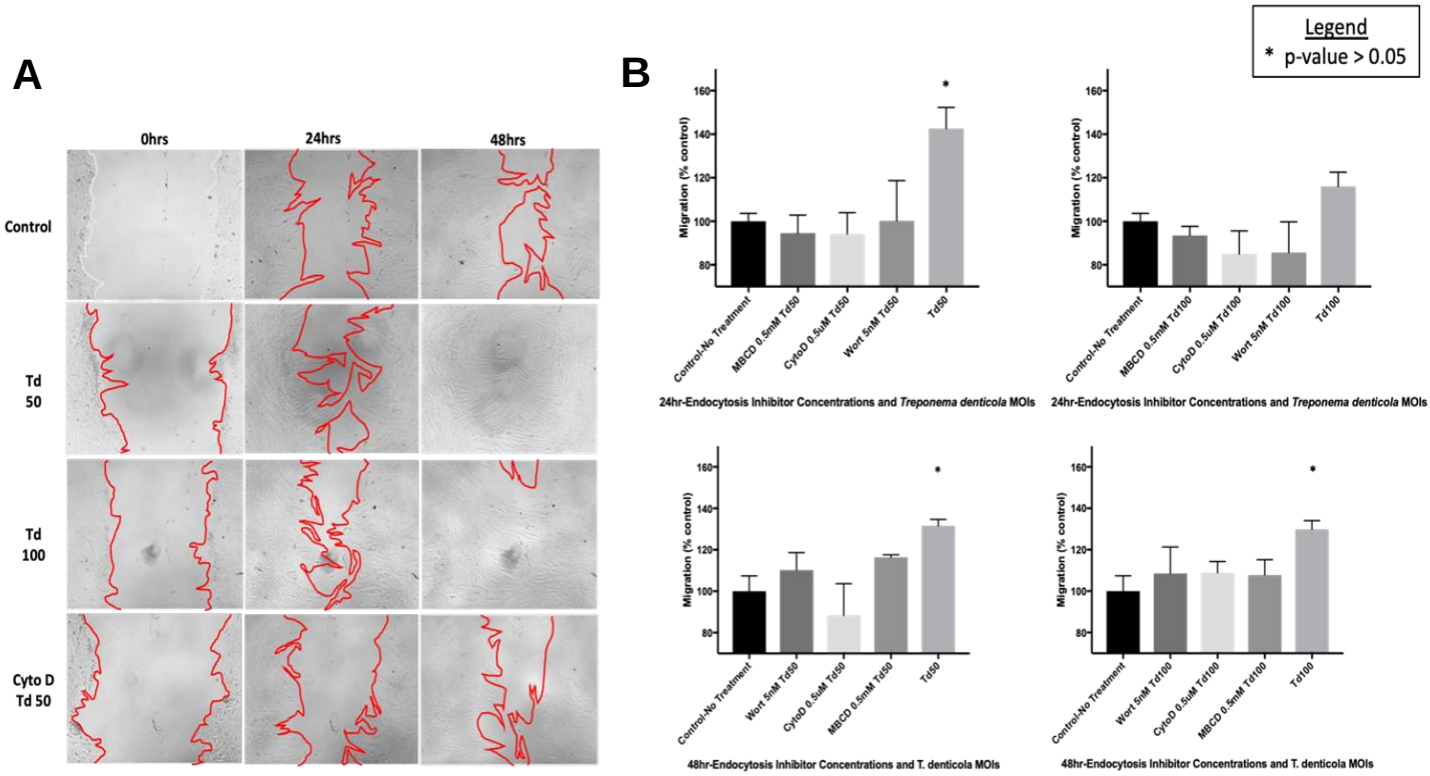


Figure 1.3.3 WT-*T. denticola* internalization enhances migration in Periodontal Ligament Cells and this effect is abrogated by an endocytosis inhibitors. Cells were challenged with WT-*T. denticola* alone or with an endocytosis inhibitor (Wortmannin, Cytochalasin D, and Methyl-Beta Cyclodextrin) at MOI: 50 and 100) for 2 hours. Inhibitors inhibit macropinocytosis (Wort), phagocytosis (CytoD), and caveolin-mediated endocytosis (MBCD). Post challenge, cells were washed with PBS to remove remaining T. denticola, incubated for 48hrs. 1.3.3 A. Light microscopy images were taken at 0hr, 24hr, and 48hr time points. 1.3.3 B. Representatives quantitative measurements of the total migratory distance of cells from edges of the wound.

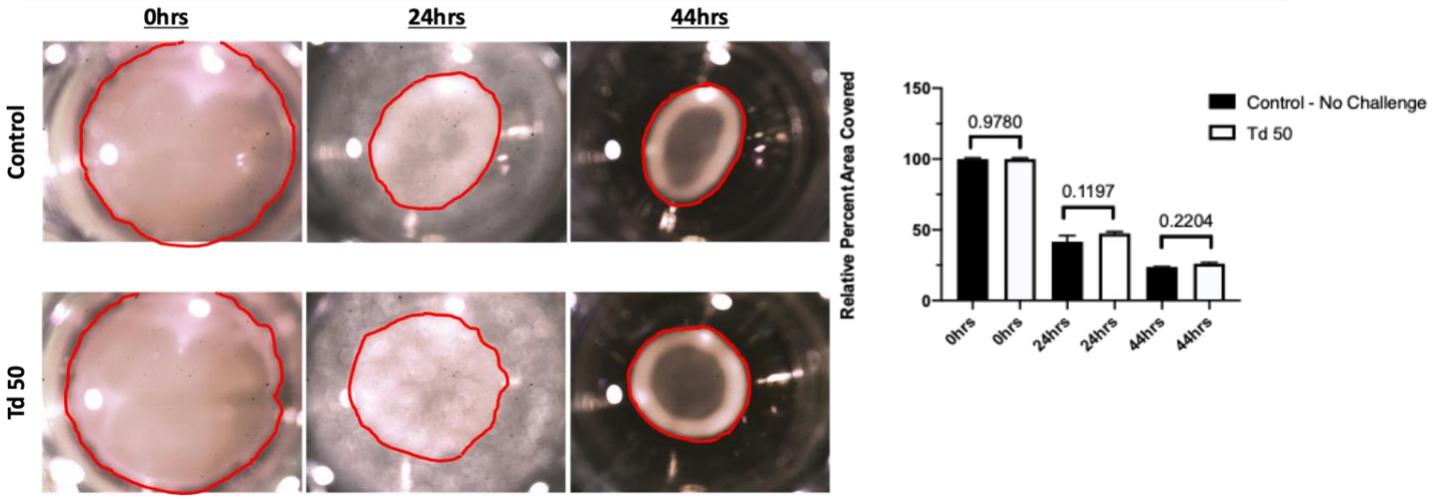


Figure 1.3.4 *Treponema denticola* does not significantly decrease hPDL ability to contract Collagen Type I. PDL cells were challenged for 2hrs and resuspended in Collagen Type I mix, then seeded in 24-well plate. Photos were taken of the contracted collagen gel on a dissecting microscope for 0hr, 24hrs, and 44hrs time points. Area of the circumference of cells plus collagen layer were calculated and compared to O hr area. Relative percent area covered was calculated for each group and plotted. FUJI imaging software was used to calculate the change in gel circumference between conditions. Student T test was used to determine significance of change between control and *Td* challenged at MOI of 50 (*Td*50) group.

Chapter 1.7: SUPPLEMENTAL FIGURES

Supplemental Table 1.7.1:

Protein Name	Sequence	AA Gap	% Identity
Dentilisin Pro-MMP-2	575 GRSYSVYGAFLGSSSNQTFTA 596 627 GHSYFFKGAYYLKLENQSLKS 647	0	33
Dentilisin Pro-MMP-2	**680 MKGG------VFQGGNYALKIDRTPLNS 701** **623 LQGGGHSYFFKGAYYLKLENQSLKS 647**	3	36
Smart00120 Pro-MMP-2	523 AD-RNYIHFYRDGRYYRMTDYGRQF 550 623 LQGGGHSYFFKGAYYLKLENQSLKS 647	3	28
Pfam00045 Pro-MMP-2	152 FQFREKAYAFCDRFYWRVSSRSELNQ 178 623 LQGGGHSYFFKGAYYLKLENQS---LKS 647	1	33

Supplemental Table 1.7.1 Blastp analysis of Dentilisin and MMP-2. The following are known domains on MMP-2, at the same location on MMP-2 where dentilisin is projected to interact.

Chapter 1.8: REFERENCES

1. Socransky, S. S., & Haffajee, A. D. (1992). The bacterial etiology of destructive periodontal disease: current concepts. Journal of periodontology, 63(4s), 322-331. (PMID:1573546)
2. Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., & Kent, R. L. (1998). Microbial complexes in subgingival plaque. Journal of clinical periodontology, 25(2), 134-144. (PMID:9495612)
3. Miao D, Fenno JC, Timm JC, Joo NE, Kapila YL. (2011). Trepenoma denticola chymotrypsin-like protease (dentisilin) induces MMP-2-dependent fibronectin fragmentation in periodontal ligament cells. Infect Immun 79:806-811. (PMC4120104)
4. Miao D, Godovikova V, Qian X, Seshadrinathan S, Kapila YL, Fenno JC. (2014). *Treponema denticola* upregulates MMP-2 activation in periodontal ligament cells: Interplay between epigenetics and periodontal infection. Arch Oral Biol 59:1056-1064. (PMC4120104)
5. Shin J.M., Ateia I., Paulus J.R., Liu H., Fenno J.C., Rickard A.H. and Kapila Y.L. Antimicrobial nisin acts against saliva derived multi-species biofilms without cytotoxicity to human oral cells. Front Microbiol. 2015 6:617 (PMC4471743)
6. Scannapieco, F. A., & Cantos, A. (2016). Oral inflammation and infection, and chronic medical diseases: implications for the elderly. Periodontology 2000, 72(1), 153-175. (PMID:27501498)
7. Kumar, P. S. (2017). From focal sepsis to periodontal medicine: a century of exploring the role of the oral microbiome in systemic disease. The Journal of physiology, 595(2), 465-476. (PMC5233655)
8. Koo BH, Park MY, Jeon OH, Kim DS. (2009) Regulatory mechanism of matrix metalloprotease-2 enzymatic activity by factor Xa and thrombin. J Biol Chem. 284(35):23375-85. doi: 10.1074/jbc.M109.036848. Epub 2009 Jul 6. (PMC2749111)

9. Chukkapalli S.S., Rivera M.F., Velsko I.M., Lee J.Y., Chen H., Zheng D., Bhattacharyya I., Gangula P.R., Lucas A.R., Kesavalu L. (2014) Invasion of oral and aortic tissues by oral spirochete *Treponema denticola* in ApoE(-/-) mice causally links periodontal disease and atherosclerosis. Infect Immun. 82(5):1959-67. doi: 10.1128/IAI.01511-14. Epub 2014 Feb 24. (PMC3993427)

10. Listgarten, M. A., & Levin S. (1981). Positive correlation between the proportions of subgingival spirochetes and motile bacteria and susceptibility of human subjects to periodontal deterioration. Journal of clinical periodontology, 8(2), 122-138. (PMC1594669)

11. Katsuji Okuda, Kazuyuki Ishihara, Taneaki Nakagawa, Akihiko Hirayama, Yoshiyuki Inayama, and Kenji Okuda (2001) Detection of *Treponema denticola* in Atherosclerotic Lesions J Clin Microbiol. 39(3): 1114–1117. doi: 10.1128/JCM.39.3.1114-1117.2001 (PMCID: PMC87882)

12. Velsko, I. M., Chukkapalli, S. S., Rivera-Kweh, M. F., Zheng, D., Aukhil, I., Lucas, A. R., Larjava, H. & Kesavalu, L. (2015). Periodontal pathogens invade gingiva and aortic adventitia and elicit inflammasome activation in αvβ6 integrin-deficient mice. Infection and immunity, 83(12), 4582-4593. (PMC4645375)

13. Fenno JC, Hannam PM, Leung WK, Tamura M, Uitto-V-J, McBride BC. (1998). Cytopathic effects of the major surface protein (Msp) and the chymotrypsine-like protease (CTLP) of *Treponema Denticola*. Infect Imunn 66:1869-1877 (PMC108137)

14. Lee, S. F., Andrian, E., Rowland, E., & Marquez, I. C. (2009). Immune response and alveolar bone resorption in a mouse model of *Treponema denticola* infection. Infection and immunity, 77(2), 694-698. doi:10.1128/IAI.01004-08

15. Isaka J1, Ohazama A, Kobayashi M, Nagashima C, Takiguchi T, Kawasaki H, Tachikawa T, Hasegawa K. (2001) Participation of periodontal ligament cells with regeneration of alveolar bone. J Periodontol. 72(3):314-23. (PMID:11327058)

16. Sculean A1, Nikolidakis D, Schwarz F. (2008) Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials - biological foundation and preclinical evidence: a systematic review. J Clin Periodontol. 35(8 Suppl):106-16. doi: 10.1111/j.1600-051X.2008.01263.x. (PMID:18724845)

17. Bildyug, N. (2016). "Matrix metalloproteinases: an emerging role in regulation of actin microfilament system." Biomol Concepts **7**(5-6): 321-329.

18. Laronha H., Caldeira J. Structure and Function of Human Matrix Metalloproteinases. Cells. 2020;9:1076. doi: 10.3390/cells9051076.

19. Franco, C., Patricia, H. R., Timo, S., Claudia, B., & Marcela, H. (2017). Matrix Metalloproteinases as Regulators of Periodontal Inflammation. *International journal of molecular sciences*, *18*(2), 440. https://doi.org/10.3390/ijms18020440

20. Checchi, V., Maravic, T., Bellini, P., Generali, L., Consolo, U., Breschi, L., & Mazzoni, A. (2020). The Role of Matrix Metalloproteinases in Periodontal Disease. *International journal of environmental research and public health*, *17*(14), 4923. https://doi.org/10.3390/ijerph17144923

21. Kamarajan, P., Garcia-Pardo, A., D'Silva, N. J., & Kapila, Y. L. (2010). The CS1 segment of fibronectin is involved in human OSCC pathogenesis by mediating OSCC cell spreading, migration, and invasion. BMC cancer, 10(1), 330. (PMC3146068)

22. Ateia I. M, Sutthiboonyapan P., Kamarajan P., Jin T., Godovikova V., Fenno J.C., Kapila Y.L. (2017). *Treponema denticola* increases MMP-2 expression via DNA and histone modifications that can be therapeutically reversed PLoS ONE; (under review)

23. Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402. (PMC146917)

24. Altschul S.F., Wootton J.C., Gertz E.M., Agarwala R., Morgulis A., Schäffer A.A., Yu Y. (2005). Protein database searches using compositionally adjusted substitution matrices, FEBS J. 272:5101-5109. (PMC1343503)

25. Gomis-Rüth FX1, Gohlke U, Betz M, Knäuper V, Murphy G, López-Otín C, Bode W. (1996). The helping hand of collagenase-3 (MMP-13): 2.7 A crystal structure of its C-terminal haemopexin-like domain. J Mol Biol. 264(3):556-66. (PMID8969305)

26. Bigg HF, Shi YE, Liu YE, Steffensen B, Overall CM. (1997). Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A. TIMP-4 binds progelatinase A and the COOH-terminal domain in a similar manner to TIMP-2. J Biol Chem. 272(24):15496-500. doi: 10.1074/jbc.272.24.15496

27. Tolosano E, Altruda F. (2002). Hemopexin: structure, function, and regulation. DNA Cell Biol. 21(4):297-306. (PMID:12042069)

28. Das S, Mandal M, Chakraborti T, Mandal A, Chakraborti S. (2003). Structure and evolutionary aspects of matrix metalloproteinases: a brief overview. Mol Cell Biochem. 253(1-2):31-40. (PMID:14619953) Shelton, L., & Rada, J. S. (2007). Effects of cyclic mechanical stretch on extracellular matrix synthesis by human scleral fibroblasts. Experimental eye research, 84(2), 314-322. (PMCID:PMC2583333)

29. Huang W1, Meng Q, Suzuki K, Nagase H, Brew K. (1997). Mutational study of the amino-terminal domain of human tissue inhibitor of metalloproteinases 1 (TIMP-1) locates an inhibitory region for matrix metalloproteinases. J Biol Chem. 272(35):22086-91. (PMID:9268350)

30. Battikhi T., Lee W., McCulloch C.A., Ellen R.P. (1999). *Treponema denticola* outer membrane enhances the phagocytosis of collagen-coated beads by gingival fibroblasts. Infect Immun. 67(3):1220- 6. (PMID:10024564)

31. Malone, E.; Ganther, S.; Mena, N.; Radaic, A.; Shariati, K.; Kindberg, A.; Tafolla, C.; Kamarajan, P.; Fenno, J.; Zhan, L.; et al. *Treponema Denticola*-Induced RASA4 Upregulation Mediates Cytoskeletal Dysfunction and MMP-2 Activity in Periodontal Fibroblasts. Front. Cell. Infect. Microbiol. 2021, 11, 671968, doi:10.3389/fcimb.2021.671968.

32. Ganther, S.; Radaic, A.; Malone, E.; Kamarajan, P.; Chang, N.-Y.N.; Tafolla, C.; Zhan, L.; Fenno, J.C.; Kapila, Y.L. *Treponema Denticola* Dentilisin Triggered TLR2/MyD88 Activation Upregulates a Tissue Destructive Program Involving MMPs via Sp1 in Human Oral Cells. PLoS Pathog. 2021, 17, e1009311, doi:10.1371/journal.ppat.1009311.

33. Kamarajan, P., Ateia, I., Shin, J. M., Fenno, J. C., Le, C., Zhan, L., Chang, A., Darveau, R., & Kapila, Y. L. (2020). Periodontal pathogens promote cancer aggressivity via TLR/MyD88 triggered activation of Integrin/FAK signaling that is therapeutically reversible by a probiotic bacteriocin. PLoS pathogens, 16(10), e1008881. https://doi.org/10.1371/journal.ppat.1008881

34. Polimeni G, Xiropaidis AV, Wikesjö UM. Biology and principles of periodontal wound healing/regeneration. Periodontol 2000. 2006;41:30-47. doi: 10.1111/j.1600-0757.2006.00157.x. PMID: 16686925.

35. Kokubu, E., Kikuchi, Y., Okamoto-Shibayama, K., & Ishihara, K. (2022). Effect of *Treponema Denticola* Infection on Epithelial Cells. The Bulletin of Tokyo Dental College, 63(1), 13–22. https://doi.org/10.2209/tdcpublication.2021-0037

36. Chi, B., Qi, M., & Kuramitsu, H. K. (2003). Role of dentilisin in *Treponema denticola* epithelial cell layer penetration. Research in microbiology, 154(9), 637–643. https://doi.org/10.1016/j.resmic.2003.08.001

37. Ramenzoni, L. L., Russo, G., Moccia, M. D., Attin, T., & Schmidlin, P. R. (2019). Periodontal bacterial supernatants modify differentiation, migration and inflammatory cytokine expression in human periodontal ligament stem cells. PloS one, 14(7), e0219181. https://doi.org/10.1371/journal.pone.0219181

38. Sarker, H., Hardy, E., Haimour, A., Maksymowych, W. P., Botto, L. D., & Fernandez-Patron, C. (2019). Identification of fibrinogen as a natural inhibitor of MMP-2. Scientific reports, 9(1), 4340. https://doi.org/10.1038/s41598-019-40983-y

39. Hinz B. (2013). Matrix mechanics and regulation of the fibroblast phenotype. Periodontology 2000, 63(1), 14–28. https://doi.org/10.1111/prd.12030

40. McCulloch, C. A., Lekic, P., & McKee, M. D. (2000). Role of physical forces in regulating the form and function of the periodontal ligament. Periodontology 2000, 24, 56–72. https://doi.org/10.1034/j.1600-0757.2000.2240104.x

41. Jun, H. K., Lee, S. H., Lee, H. R., & Choi, B. K. (2012). Integrin α5β1 activates the NLRP3 inflammasome by direct interaction with a bacterial surface protein. Immunity, 36(5), 755–768. https://doi.org/10.1016/j.immuni.2012.05.002

42. Li, M., Liu, Z., & Umemoto, T. (1999). Zhonghua kou qiang yi xue za zhi = Zhonghua kouqiang yixue zazhi = Chinese journal of stomatology, 34(3), 165–167.

43. Umemoto, T., Li, M., & Namikawa, I. (1997). Adherence of human oral spirochetes by collagen-binding proteins. Microbiology and immunology, 41(12), 917–923. https://doi.org/10.1111/j.1348-0421.1997.tb01950.x

44. Batista da Silva, A. P., Lee, W., Bajenova, E., McCulloch, C. A., & Ellen, R. P. (2004). The major outer sheath protein of *Treponema denticola* inhibits the binding step of collagen phagocytosis in fibroblasts. Cellular microbiology, 6(5), 485–498. https://doi.org/10.1111/j.1462-5822.2004.00377.x

45. Amin, M., A. C. Ho, J. Y. Lin, A. P. Batista da Silva, M. Glogauer and R. P. Ellen (2004). "Induction of de novo subcortical actin filament assembly by *Treponema denticola* major outer sheath protein." Infect Immun 72(6): 3650-3654.

46. Hočevar K, Potempa J, Turk B. Host cell-surface proteins as substrates of gingipains, the main proteases of *Porphyromonas gingivalis*. Biol Chem. 2018;399(12):1353–1361. doi: 10.1515/hsz-2018-0215.

47. Murakami Y. Fibronectin in saliva inhibits *Porphyromonas gingivalis* fimbria-induced expression of inflammatory cytokine gene in mouse macrophages. FEMS Immunol Med Microbiol. 1998;22(3):257–262. doi: 10.1016/S0928-8244(98)00098-4.

48. Al-Taweel FBH, Al-Magsoosi MJN, Douglas CWI, Whawell SA. Identification of key determinants in *Porphyromonas gingivalis* host-cell invasion assays. Eur J Oral Sci. 2018;126(5):367–372. doi: 10.1111/eos.12557.

49. Stafford, P., Higham, J., Pinnock, A., Murdoch, C., Douglas, C. W., Stafford, G. P., & Lambert, D. W. (2013). Gingipain-dependent degradation of mammalian target of rapamycin pathway proteins by the periodontal pathogen *Porphyromonas gingivalis* during invasion. Molecular oral microbiology, 28(5), 366–378. https://doi.org/10.1111/omi.12030

50. Tanner A, Maiden MFJ, Macuch PJ, Murray LL, Kent RL. Microbiota of health, gingivitis, and initial periodontitis. J Clin Periodontol. 1998;25(2):85–98. doi: 10.1111/j.1600-051X.1998.tb02414.x.

51. Ksiazek M, Karim AY, Bryzek D, et al. (2015) Mirolase, a novel subtilisin-like serine protease from the periodontopathogen Tannerella forsythia. Biol Chem. 396(3). doi:10.1515/hsz-2014-0256

52. Marre, A. T. O., Domingues, R. M. C. P., & Lobo, L. A. (2020). Adhesion of anaerobic periodontal pathogens to extracellular matrix proteins. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology], 51(4), 1483–1491. https://doi.org/10.1007/s42770-020-00312-2

53. Sharma A, Sojar HT, Glurich I, Honma K, Kuramitsu HK, Genco RJ (1998) Cloning, expression, and sequencing of a cell surface antigen containing a leucine-rich repeat motif from Bacteroides forsythus ATCC 43037. McGhee JR, ed. Infect Immun 66(12):5703–5710. doi:10.1128/IAI.66.12.5703-5710.1998

54. Abe T, Murakami Y, Nagano K, Hasegawa Y, Moriguchi K, Ohno N, Shimozato K, Yoshimura F. OmpA-like protein influences cell shape and adhesive activity of Tannerella forsythia. Mol Oral Microbiol. 2011;26(6):374–387. doi: 10.1111/j.2041-1014.2011.00625.x.

55. Karim, A. Y., Kulczycka, M., Kantyka, T., Dubin, G., Jabaiah, A., Daugherty, P. S., Thogersen, I. B., Enghild, J. J., Nguyen, K. A., & Potempa, J. (2010). A novel matrix metalloprotease-like enzyme (karilysin) of the periodontal pathogen *Tannerella forsythia* ATCC 43037. Biological chemistry, 391(1), 105–117. https://doi.org/10.1515/BC.2010.009

56. Cerdà-Costa, N., Guevara, T., Karim, A. Y., Ksiazek, M., Nguyen, K. A., Arolas, J. L., Potempa, J., & Gomis-Rüth, F. X. (2011). The structure of the catalytic domain of *Tannerella forsythia* karilysin reveals it is a bacterial xenologue of animal matrix metalloproteinases. Molecular microbiology, 79(1), 119–132. https://doi.org/10.1111/j.1365-2958.2010.07434.x

57. López-Pelegrín, M., Ksiazek, M., Karim, A. Y., Guevara, T., Arolas, J. L., Potempa, J., & Gomis-Rüth, F. X. (2015). A novel mechanism of latency in matrix metalloproteinases. The Journal of biological chemistry, 290(8), 4728–4740. https://doi.org/10.1074/jbc.M114.605956

58. Edwards AM, Jenkinson HF, Woodward MJ, Dymock D. Binding properties and adhesion-mediating regions of the major sheath protein of *Treponema denticola* ATCC 35405. Infect Immun. 2005;73(5):2891–2898. doi: 10.1128/IAI.73.5.2891-2898.2005.

59. Fenno JC, Tamura M, Hannam PM, Wong GWK, Chan RA, McBride BC. Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. Infect Immun. 2000;68(4):1884–1892. doi: 10.1128/IAI.68.4.1884-1892.2000.

60. Bamford CV, Fenno JC, Jenkinson HF, Dymock D. The chymotrypsin-like protease complex of *Treponema denticola* ATCC 35405 mediates fibrinogen adherence and degradation. Infect Immun. 2007;75(9):4364–4372. doi: 10.1128/IAI.00258-07.

61. Nussbaum G, Ben-Adi S, Genzler T, Sela M, Rosen G. 2009. Involvement of Toll-like receptors 2 and 4 in the innate immune response to *Treponema denticola* and its outer sheath components. Infect Immun 77:3939–3947. doi: 10.1128/IAI.00488-09.

62. Opitz B, Schroder NW, Spreitzer I, Michelsen KS, Kirschning CJ, Hallatschek W, Zahringer U, Hartung T, Gobel UB, Schumann RR. 2001. Toll-like receptor-2 mediates Treponema glycolipid and lipoteichoic acid-induced NF-κB translocation. J Biol Chem 276:22041–22047. doi: 10.1074/jbc.M010481200

63. Ruby, J., Martin, M., Passineau, M. J., Godovikova, V., Fenno, J. C., & Wu, H. (2018). Correction for Ruby et al., "Activation of the Innate Immune System by *Treponema denticola* Periplasmic Flagella through Toll-Like Receptor 2". Infection and immunity, 86(7), e00251-18. https://doi.org/10.1128/IAI.00251-18

64. Nieminen, M. T., Listyarifah, D., Hagström, J., Haglund, C., Grenier, D., Nordström, D., Uitto, V. J., Hernandez, M., Yucel-Lindberg, T., Tervahartiala, T., Ainola, M., & Sorsa, T. (2018). *Treponema denticola* chymotrypsin-like proteinase may contribute to orodigestive carcinogenesis through immunomodulation. British journal of cancer, 118(3), 428–434. https://doi.org/10.1038/bjc.2017.409

65. Uitto, V. J., Y. M. Pan, W. K. Leung, H. Larjava, R. P. Ellen, B. B. Finlay, and B. C. McBride. 1995. Cytopathic effects of *Treponema denticola* chymotrypsin-like proteinase on migrating and stratified epithelial cells. Infect. Immun. 63:3401-3410.

66. Verma, R. K., Rajapakse, S., Meka, A., Hamrick, C., Pola, S., Bhattacharyya, I., Nair, M., Wallet, S. M., Aukhil, I., & Kesavalu, L. (2010). *Porphyromonas gingivalis* and *Treponema denticola* Mixed Microbial Infection in a Rat Model of Periodontal Disease. Interdisciplinary perspectives on infectious diseases, 2010, 605125. https://doi.org/10.1155/2010/605125

67. Godovikova, V., H. T. Wang, M. P. Goetting-Minesky, Y. Ning, R. F. Capone, C. K. Slater and J. C. Fenno (2010). "*Treponema denticola* PrcB is required for expression and activity of the PrcA-PrtP (dentilisin) complex." J Bacteriol 192(13): 3337-3344.

68. Kapila, Y. L., Kapila, S., & Johnson, P. W. (1996). Fibronectin and fibronectin fragments modulate the expression of proteinases and proteinase inhibitors in human periodontal

ligament cells. *Matrix biology : journal of the International Society for Matrix Biology*, *15*(4), 251–261. https://doi.org/10.1016/s0945-053x(96)90116-x

69. Al-Rekabi, Z., Fura, A. M., Juhlin, I., Yassin, A., Popowics, T. E., & Sniadecki, N. J. (2019). Hyaluronan-CD44 interactions mediate contractility and migration in periodontal ligament cells. *Cell adhesion & migration*, *13*(1), 138–150. https://doi.org/10.1080/19336918.2019.1568140

Chapter 2: *Treponema denticola*-Induced RASA4 Upregulation Mediates Cytoskeletal Dysfunction and MMP-2 Activity in Periodontal Fibroblasts

Chapter 2.1 ABSTRACT

The periodontal complex consists of the periodontal ligament (PDL), alveolar bone, and cementum, which work together to turn mechanical load into biological responses that are responsible for maintaining a homeostatic environment. However oral microbes, under conditions of dysbiosis, may challenge the actin dynamic properties of the PDL in the context of periodontal disease. To study this process, we examined host-microbial interactions in the context of the periodontium via molecular and functional cell assays and showed that human PDL cell interactions with *Treponema denticola* induce actin depolymerization through a novel actin reorganization signaling mechanism. This actin reorganization mechanism and loss of cell adhesion is a pathological response characterized by an initial upregulation of RASA4 mRNA expression resulting in an increase in matrix metalloproteinase-2 activity. This mechanism is specific to the *T. denticola* effector protein, dentilisin, thereby uncovering a novel effect for *Treponema denticola*-mediated RASA4 transcriptional activation and actin depolymerization in primary human PDL cells.

Chapter 2.2 INTRODUCTION

Periodontal disease is characterized by an altered periodontal ligament space, chronic inflammation, and destruction of the periodontal tissues, including a breakdown of the extracellular matrix (ECM) (Armitage 2004). The periodontal complex consists of the periodontal ligament (PDL), alveolar bone, and cementum, which work together to turn mechanical load into biological responses that are responsible for maintaining a homeostatic environment (Jang, Chen et al. 2018). Cytopathic mechanisms in mechanotransduction are facilitated by cytoskeletal interaction with the ECM (Humphrey, et al. 2014) and include ECM degradation and remodeling (Tsuji, Uno et al. 2004, Manokawinchoke, Limjeerajarus et al. 2015), altered cellular differentiation (Kanzaki, Chiba et al. 2002), altered cellular migration, and altered cellular adhesion or de-adhesion processes (Kang, Nam et al. 2010, Wang, Davidson et al. 2019). A failure of mechanotransduction and actin organization within the periodontium results in loss of regeneration of periodontal tissues, remodeling of the ECM, and progression of disease; processes highly regulated within the PDL. (Chukkapalli, Lele et al. 2018) The disease process is initiated by pathogenic microbes under conditions of microbial dysbiosis. *Treponema denticola*, an oral spirochete identified as a periodontal pathogen, is implicated in this dysbiosis leading to chronic inflammation, ECM remodeling, including enhanced MMP-2 activation, and periodontal tissue destruction (Takeuchi, Umeda et al. 2001, Miao, Fenno et al. 2011, Miao, Godovikova et al. 2014, Ruby, Martin et al. 2018).

Actin monomer and filament dynamics are necessary for mechanotransduction and are capable of regulating epigenetic enzymes (Sadhukhan, Sarkar et al. 2014, Serebryannyy, Cruz et al. 2016), chromatin reprogramming (Zhao, Wang et al. 1998, Kapoor and Shen 2014), transcriptional machinery (Visa and Percipalle 2010), and gene expression (Misu, Takebayashi et al. 2017). Mechanotransduction and actin dynamics can also influence homeostatic conditions towards disease through extracellular process and tissue destructive mechanisms (Humphrey, Dufresne et al. 2014). Actin dynamics are also involved in the regulation of tissue destructive genes, like matrix metalloproteinases (MMP) (Bildyug

2016). MMP-2 is overexpressed in periodontal tissues compromised by apical periodontitis (Fernández, Cárdenas et al. 2019), chronic apical abscesses (Letra, Ghaneh et al. 2013), and chronic periodontitis (Ateia, Sutthiboonyapan et al. 2018); implicating MMP-2 regulation in periodontitis. Major periodontopathogens, like *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Actinobacillus actinomycetemcomitans* can induce actin reorganization, thereby providing a possible mechanism through which microbial infection can facilitate periodontitis (Hassell, Baehni et al. 1997, Yilmaz, Young et al. 2003, Gutiérrez-Venegas, Kawasaki-Cárdenas et al. 2007, Hasegawa, Tribble et al. 2008, Kinane, Benakanakere et al. 2012, Zhang, Ju et al. 2013). *T. denticola* is also capable of affecting filamentous actin abundance in human gingival fibroblasts and epithelial cells (Baehni, Song et al. 1992, Yang, Song et al. 1998). Previous literature suggests that *T. denticola* induces subcortical actin polymerization through its effector protein, major surface protein (Wang, Ko et al. 2001, Amin, Ho et al. 2004, Visser, Koh et al. 2011). However, little is known regarding *T. denticola's ability to* affect the actin dynamic function of human PDL cells (PDL) and the signaling components involved in the host response to *T. denticola* and effector protein dentilisin. In the present study, we characterize a novel mechanism demonstrating *T. denticola*-induced actin remodeling through stimulation of the Ca (2+)-dependent Ras GTPase-activating protein, RASA4 that further regulates MMP-2 activity. The overall objective of this study was to determine *T. denticola's* influence on PDL cellular actin dynamics and the mechanism of action that may contribute to tissue destruction, including activation of MMP-2.

Chapter 2.3 RESULTS

2.3.1 PDL cell attachment and contractility is impaired by *T. denticola* challenge

Since little is known about *T. denticola's* effects on the actin filament processes of human PDL cells (PDL), we began this investigation by analyzing *T. denticola's* modulation of cellular adhesion and live actin filament dynamics in PDL cells (Figure 1 and Supporting Information, Figure 1 video). *T. denticola* interactions with gingival fibroblasts disrupt cell adhesion (Baehni, Song et al. 1992), therefore we examined *T. denticola's* effects on PDL cell adhesion. *T. denticola* challenged PDL cells exhibited 70% more detachment than unchallenged cells (Figure 1). Live imaging of *T. denticola* challenged cells (Supporting Information, Figure 1 and video) showed an impaired contractility leading to detachment of cells. Together, this indicates that *T. denticola* interaction with PDL cells negatively influences their actin dynamic properties. PDL cell interactions with *T. denticola* at an MOI of 50 were not cytotoxic to PDL cells as assessed with a Calcein AM viability assay (Supporting Information, Figure 2).

2.3.2 T. denticola decreases stress fiber and β-actin monomer abundance in PDL cells

Actin stress fibers are key to the mechanotransducing function of PDL cells (de Araujo, Oba et al. 2014). Therefore, we next analyzed *T. denticola's* modulation of actin stress fibers and actin monomers in PDL cells (Figure 2). Treatment of PDL cells with *T. denticola* resulted in a significant decrease in stress fiber abundance as evaluated with immunofluorescence staining (Figure 2A). A 30% reduction in mean optical density was noted. To further evaluate *T. denticola's* effect on actin fibers, β-actin protein expression was evaluated in this context. Western blotting analyses revealed that *T. denticola* reduced β-actin protein levels by approximately 30% in PDL cells (Figure 2B). RNA sequencing analyses further revealed that *T. denticola* also significantly decreased actin RNA levels in PDL cells at 5 and 24 hours by 60 and 76%, respectively (Figure 2C). Additionally, gene ontology enrichment analysis revealed a significant

differential expression in the biological pathways related to actin and cytoskeletal organization within the 5 h challenge samples compared to the controls (Supporting Information, Figure 3).

2.3.3 Gene pathway and volcano plot analyses show RASA4 as a top upregulated gene in *T. denticola* challenged PDL cells

From the RNA sequencing data referenced previously, gene ontology (GO) enrichment data was derived for 5 h and 24 h challenged samples versus controls (Supporting Information, Figure 3; Figure 3). Among all biological pathways, actin and cytoskeletal regulatory pathways were significantly and differentially expressed at 5 h after *T. denticola* challenge. Actin cytoskeletal-related pathways were also among the top significantly and differentially expressed biological pathways, including the ras protein signal transduction and regulation of small GTPase mediated signal transduction pathways. In order to identify the top differentially expressed genes that were influenced by actin regulation and that could be upstream of MMP-2 regulation, we plotted genes that were identified within the significantly expressed biological processes from the gene ontology (Figure 3A). From these genes, RASA4 was identified as one of the top genes upregulated upon a 24 h *T. denticola* challenge. Since specific tissues are able to regulate MMP-2 activity via actin dynamics (Bildyug, 2016), we investigated the role of RASA4, as RASA4 is linked to actin reorganization and stress fiber maintenance. Interestingly, RASA4 was upregulated 1.7-fold in the *T. denticola*-challenged cells compared to controls (Figure 3B).

2.3.4 Purified dentilisin and *T. denticola* upregulate RASA4 gene expression in PDL cells

Previous literature suggests that *T. denticola* induces subcortical actin polymerization through its effector protein, major surface protein (Msp) (Visser, Koh et al. 2011, Wang, Davidson et al. 2019). Purified Msp can induce actin assembly by blocking the calcium influx into fibroblasts (Wang, Ko et al. 2001). Since RASA4 is activated by a calcium influx, the *T. denticola*-mediated mechanism of action in our system

may be different. Therefore, we investigated the role of another *T. denticola* effector protein, dentilisin. We used purified dentilisin and a *T. denticola* isogenic mutant (CF522-*Td*; dentilisin null and Msp positive), which lacks the proteolytic PrtP subunit of dentilisin to determine if dentilisin was sufficient to enhance RASA4 upregulation. Additionally, we tested the *T. denticola* MHE strain (an isogenic Msp mutant that is dentilisin-positive) to investigate the role of Msp in this process. *Veillonella parvula* was used as an additional control, since it is a commensal Gram-negative bacterium. Quantitative RT-PCR results indicate that both purified dentilisin and wild type-*T. denticola* induced RASA4 expression (1.7 and 1.67-fold increase, respectively) (Figure 4). Neither CF522-*Td*, MHE-*Td* nor *V. parvula* had any effect on RASA4 expression compared to untreated PDL cells (Figure 4). Thus, our results indicate that while dentilisin contributes to *T. denticola*-mediated RASA4 transcriptional upregulation, Msp activity also contributes, presumably by a different mechanism.

2.3.5 Jasplakinolide inhibits *T. denticola*-induced RASA4 gene expression, whereas RASA4 gene expression is upregulated in Latrunculin B pretreated and *T. denticola* challenged PDL cells.

T. denticola causes actin reorganization in fibroblasts (Visser, Koh et al. 2011). In this study, we showed that *T. denticola* decreases actin stress fibers in PDL cells (F-actin intensity; Figure 2). Previous literature showed that RASA4 can disrupt actin stress fiber organization (Choi and Helfman 2014) and induce actin reorganization (Choi and Helfman 2014, Li, Zeng et al. 2017). We hypothesized that RASA4 would also be upregulated during actin depolymerization but not during polymerization. Jasplakinolide (Jasp) or Latrunculin B (Lat B) were used to pre-treat PDL cells to induce actin polymerization or depolymerization, respectively. Post-treatment cells were collected for reverse transcriptase-quantitative PCR analysis. Results showed that Jasplakinolide did not have an effect on RASA4 gene expression compared to controls (Figure 5A). We next investigated the effect of actin depolymerization (which was induced by Latrunculin B pretreatment) on RASA4 gene expression. RASA4 gene expression was significantly upregulated (1.67 fold) with *T. denticola* and (2.4 fold) with Latrunculin B treatment compared to controls

(Figure 5B). Combined treatment of Latrunculin B and *T. denticola* further upregulated RASA4 gene expression (4.15 fold). Thus, RASA4 upregulation is associated with *T. denticola*-mediated actin depolymerization and the actin depolymerization agent Latrunculin B.

2.3.6 RASA4 gene expression is important for *T. denticola*-mediated actin stress fiber dysfunction

To further determine the role of RASA4 in *T. denticola*-mediated actin depolymerization, we suppressed RASA4 gene expression in this context. RASA4 expression was reduced by 60% in hPDL cells infected with RASA4 shRNA lentiviral particles compared to control infected cells (Figure 6A). These cells were then challenged with or without *T. denticola*. After challenge, cells were stained with actin to visualize cellular stress fiber abundance by confocal microscopy. PDL cells infected with shRNA RASA4 exhibited reduced stress fiber abundance and organization compared to control cells treated with scrambled shRNA (Scr shRNA) (Figure 6B). Image analysis of actin stress fiber staining intensity showed that shRNA RASA4 infected cells exhibited a 2.4-fold decrease compared to control infected cells (Figure 6C). In contrast, *T. denticola* challenge did not change the levels of actin staining intensity any further. These data indicate that RASA4 gene expression is important for homeostatic actin organization and for *T. denticola*-mediated depolymerization of stress fibers in PDL cells.

2.3.7 Actin depolymerization through *T. denticola* challenge and Latrunculin B pretreatment increases MMP-2 enzymatic activity

Actin dynamics have been shown to regulate MMPs (Bildyug, 2016). Additionally, we previously showed that MMP-2 activation increased upon *T. denticola* challenge (Miao, Godovikova et al. 2014). Thus, we investigated whether *T. denticola*-mediated changes in actin dynamics could modulate MMP-2 activity in PDL cells and if the actin-depolymerizing effect of the chemical inhibitor Latrunculin B (Lat B) would affect MMP expression in this context. As in Figure 5, Latrunculin B (Lat B) or Jasplakinolide (Jasp) were used

to pre-treat PDL cells to induce actin depolymerization or polymerization, respectively. Following pre-treatment, *T. denticola* challenged PDL cells were collected for gelatin zymography to analyze MMP-2 activity (Figure 7A). There were no significant changes in the inactive form of MMP-2 (pro-MMP-2) among any of the samples (Figure 7B). Induction of actin polymerization with Jasplakinolide did not result in any difference in MMP-2 activation compared to control (Figure 7C). Challenging PDL cells with *T. denticola* alone significantly increased MMP-2 activity 2.8-fold, but pre-treatment with Jasplakinolide before *T. denticola* challenge decreased MMP-2 activity (1.6 fold compared to control), thereby abrogating the effect of *T. denticola* on MMP-2 (Figure 7C). In contrast, induction of actin depolymerization in PDL cells with Latrunculin B alone showed a notable increase in active MMP-2 bands (2.8-fold) compared to control (Figure 7C). Additionally, samples pretreated with Latrunculin B, then challenged with *T. denticola*, also increased MMP-2 activity (3.2 fold) compared to control. These data indicate that actin dynamics regulate MMP-2 activity, and *T. denticola* mediates these MMP-2 effects through an actin depolymerization mechanism.

2.3.8 RASA4 gene expression is required for *T. denticola*-mediated enhancement of MMP-2 activity

Since RASA4 is required for *T. denticola*-mediated actin depolymerization and since actin dynamics mediate changes in MMP, we next investigated whether RASA4 is required for *T. denticola*-mediated changes in MMP-2 activity in PDL cells. Using the same experimental set up as in Figure 6, we obtained culture media from each group to analyze MMP-2 activation. Gelatin zymography of culture media from these cells showed that *T. denticola* challenge significantly increased active MMP-2 levels (2.5 fold) in controls (Scr shRNA cells plus *T. denticola)* compared to unchallenged (Scr shRNA) cells (Figure 8A-C). In contrast, shRNA RASA4 infected cells exhibited decreased MMP-2 activity (1.67-fold) compared to controls (Scr shRNA), and *T. denticola* was unable to increase the levels of MMP-2 activity in the context of RASA4 shRNA (Figure 8A-C). This indicates that RASA4 is required for *T. denticola* driven induction of depolymerization and subsequent regulation of MMP-2 activity in PDL cells.

Chapter 2.4: DISCUSSION

In this study, we show that in *T. denticola*-PDL cell interactions*, T. denticola* drives enhanced MMP-2 activity through a novel actin reorganization signaling mechanism. This reorganization mechanism is a pathological response characterized by upregulation of RASA4, depolymerization of actin filaments, and a subsequent increase in MMP-2 activity. This mechanism is specific to the *T. denticola* effector protein dentilisin (although Msp is also important), thereby uncovering a novel effect for *T. denticola*-mediated actin depolymerization in primary human PDL cells that is distinct from that previously reported for *T. denticola* Msp in human gingival fibroblasts. Although there are different mechanisms reported for *T. denticola*'s contribution to actin reorganization, Jobin et al. found that *T. denticola* purified Msp induces Ca(2+) entry and actin reorganization in cultured fibroblasts (Jobin, Virdee et al. 2007). This is contrary to other findings by Ko et al. who reported that outer membrane extracts of *T. denticola* inhibited calcium influxes in gingival fibroblasts and disrupted actin-dependent processes (Ko, Glogauer et al. 1998). This suggests that the bacterial source (purified versus bacterial extracts) is important in determining host effects on actin organization and calcium influxes, but knowledge of the overall effects of the whole bacterium is lacking. Furthermore, using *T. denticola* mutant strains carrying defined defects in proteins of interest to assay their biological relevance is important. Earlier literature showed that Latrunculin B treatment induced calcium influxes though that study did not assay effects of a defined Msp mutant strain (Wang, Ko et al. 2001). We show here that the calcium influx-triggered gene, RASA4, is upregulated in the presence of Latrunculin B and its expression is further increased in the presence of *T. denticola*. We also show the importance of *T. denticola* dentilisin and the Msp proteins in this process. While neither the dentilisin mutant nor the Msp mutant showed effects on RASA4 expression significantly different from controls, further studies are required to determine whether a double mutant would show a distinguishable difference. Taken in aggregate, these data indicate that dentilisin and Msp are key *T. denticola* proteins important in regulating actin assembly and calcium influx through RASA4 is mechanistically important (Ko, Glogauer et al. 1998, Jobin, Virdee et al. 2007, Visser, Koh et al. 2011).

There are various signaling pathways implicated in actin organization and stress fiber formation. *T. denticola*-mediated actin reorganization is stimulated by PIP-2 dependent signaling leading to activation of the small GTPases RAC1, RhoA, and Ras in fibroblasts (Visser, Koh et al. 2011). To further evaluate the global effects of *T. denticola* on the PDL cytoskeleton at the mRNA level, we performed RNA sequencing of PDL challenged cells. RNA sequencing analysis of *T. denticola*-challenged PDL cells compared to non-challenged cells revealed a significant differential expression of genes related to actin cytoskeletal dynamics, including the actin monomer (β-actin), γ-actin, IQGAP3, ACTR3, ACTR2, ARPC3, ARPC5, RERG, and ARHGEF4. Gene ontology also confirmed that Ras signaling was amongst the top 20 most upregulated pathways. When examining actin organization and *ras* signaling genes, a candidate gene RASA4 was identified as a key upregulated gene (increased 1.7-fold, p-value 7.62E-04) in *T. denticola* challenged cells. Its paralog RASA4B was also upregulated 1.57-fold (p-value 3.82E-02). We confirmed that *T. denticola* and its secreted protease dentilisin are responsible for RASA4 gene upregulation. This is consistent with previous reports identifying RASA4 as important for microbial immune responses to bacterial infection (Zhang, Guo et al. 2005). Our study suggests that RASA4 may be a key cellular response to periodontal infection and a possible biomarker for *Treponema denticola* and *Treponema denticola*-associated diseases.

RASA4 can constitutively interact with Rac1 and Cdc42, and it inhibits the RAS/ERK signaling pathway, which is important for maintaining actin organization. Inhibition of the RAS/ERK pathway via RASA4 disrupts actin stress fiber organization (Choi and Helfman 2014) and induces actin reorganization (Choi and Helfman 2014, Li, Zeng et al. 2017). This evidence suggests that RASA4 may be a key regulator of actin stress fiber reorganization stimulated by *T. denticola* challenge. By knocking down RASA4 and showing no difference in F-acin abundance with or without *T. denticola,* our results show that RASA4 is required for actin dynamics, specifically, actin depolymerization in the presence of *T. denticola.* Our data

is consistent with other reports showing that actin reorganization is induced by *T. denticola.* Other publications suggest that this is facilitated by the effector protein Msp (Visser, Koh et al. 2011). Given the current data with the mutant *T. denticola* strains highlighting the role of dentilisin and the importance of the Msp, it is possible that RASA4 is acting through a unique mechanism as a result of both dentilisin and the Msp.

RASA4 has not been previously linked to MMP-2 activity. Our results demonstrate that a periodontal pathogen, *T. denticola* relies on RASA4 genetic expression to induce actin stress fiber disruption and increase MMP-2 activity in PDL cells. In order to confirm that RASA4 was involved in enhanced MMP-2 activity, a suppression strategy was used. Successful suppression of RASA4 expression effectively decreased overall pro-MMP-2 activity and active MMP-2. In the presence of *T. denticola,* MMP-2 activity is normally enhanced in PDL cells (Miao, et al., 2011). Our study suggests that this increase is due to actin depolymerization of actin stress fibers, but suppression of RASA4 abrogated the effect of *T. denticola* on these actin components and MMP-2*.* These results indicate that RASA4 mRNA expression is required for actin-mediated enhanced MMP-2 activity. This study is the first to characterize RASA4 as a key player in *T. denticola*-mediated actin reorganization and MMP-2 activity in PDL cells.

Several periodontal pathogens have been reported to increase MMP-2 expression and activity. Known red and orange complex bacteria (Socransky, Haffajee et al. 1998), *Porphymonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Fusobacteria nucleatum* are able to stimulate MMP-2 production and secretion in human epithelial cells and PDL cells (Pattamapun, Tiranathanagul et al. 2003, Tiranathanagul, Pattamapun et al. 2004, Gursoy, Könönen et al. 2008). We have previously shown that *T. denticola* increases both activation of MMP-2 proteolytic activity and transcription through a chronic, epigenetic mechanism (Miao, Fenno et al. 2011, Ateia, Sutthiboonyapan et al. 2018), resulting in MMP-2-dependent fibronectin fragmentation (Miao, Fenno et al. 2011). Together, these studies suggest that

bacterial stimulation of MMP-2 and its co-activators/modulators by periodontal pathogens plays a role in tissue destruction; however, the mechanism(s) involved for individual species and host cell types are not well understood. My dissertation investigated characterizing cytopathic functions and biological pathways triggered by *T. denticola* interacting with human periodontal ligament cells.

Additionally, activators of MMP-2 are affected by *Treponema denticola* interactions. Tissue inhibitors of metalloproteinases, specifically TIMP-1 and -2, are important in the inhibition and activation of MMP-2. Previous studies have shown that *T. denticola* challenge of PDL cells upregulates TIMP-2 and MMP-14 mRNA expression. (Miao, et al 2014) Dentilisin is important in the increase in protein levels of TIMP-2 and MMP-14. (Miao, et al 2014) Furthermore, purified dentilisin can breakdown TIMP-1 and TIMP-2. (Nieminen, et al. 2018) These conditions would favor a ratio for maximum MMP-2 activation. Together, these studies suggest that bacterial stimulation of MMP-2 and its co-activators/modulators by periodontal pathogens plays a role in tissue destruction; however, the mechanism(s) involved for individual species and host cell types are not well understood. The present study examined RASA4 gene expression as a regulator for *T. denticola*-mediated enhancement of MMP-2 activity. Calcium-dependent signaling can also induce MMP-2 expression in multiple cell types, including human periodontal fibroblasts and oral squamous cell carcinoma cells (Munshi, Wu et al. 2002, Osorio, Cavalla et al. 2015). Consistent with this finding, we show that RASA4 (which relies on calcium signaling) is necessary for *T. denticola* interaction to increase MMP-2 activity. Additionally, our results indicate that *T. denticola*-mediated actin reorganization requires RASA4 gene expression. Actin reorganization regulates MMPs in different ways; showing tissue specificity (Bildyug 2016). MMP-2 expression can increase or decrease due to actin dynamics; polymerization and depolymerization (Sanka, Maddala et al. 2007, Bildyug 2016). For example, inhibition of actin polymerization had no effect on MMP-2 expression in HT1080 fibrosarcoma cells, whereas in human trabecular meshwork cells, inhibition of actin polymerization induced the activation of MMP-2 expression (Chintala, Sawaya et al. 1998, Sanka, Maddala et al. 2007). We examined whether actin dynamics are involved in our system and found that sequestering actin

monomers (which depolymerizes actin stress fibers) by treatment with latrunculin B, increased active MMP-2 activity in PDL cells. In summary, the current study highlights that *T. denticola* mediates actin reorganization (depolymerization) through RASA4 gene expression, which enhances MMP-2 activity in human PDL cells.

MMP-2 and MMP-9 are the major gelatinases amongst MMPs. *T. denticola* is associated with MMP-9 in gingival crevicular fluid from periodontally challenged patients compared to healthy patients (Yakob, et al. 2013). PMN interaction with Treponeme components, chiefly the 53-kDa protein and LPS, increased the release of MMP-9. (Ding, et al 1996) The MSP effector protein, enhances the production of MMP-9 in peripheral blood monocytes. In isolation, purified dentilisin does have the ability to convert purified proMMP-9 to active MMP-9. (Nieminen, et al. 2018) MMP-9 may play a role in the context of this paper, but PDL cells do not make large amounts of MMP-9. PDL cells are major producers of MMP-2. Clinically, this is relevant because MMP-2 plays a key role in fibronectin cleavage. The presence of 40, 68, and 120kDa fibronectin (FN) fragments in gingival crevicular fluid are markers of periodontal disease. (Huynh, et al. 2002) PDL cells create these FN fragments predominately through enhanced production of MMP-2 activity and mRNA expression in response to *T. denticola* and purified dentilisin challenge. (Huynh, et al. 2002; Miao, et al. 2011, Miao, et al. 2014) Knockdown of MMP-2 nearly eliminates FN fragments. Therefore MMP-2 was the relevant MMP in this study, but further study may elucidate MMP-9 playing a significant role. (Miao, et al 2011; Yakob, et al. 2013)

Chapter 2.5: MATERIALS AND METHODS

Human Subjects and IRB Approval

Institutional review board (IRB) approval for human subjects research was obtained via the University of California San Francisco institutional review board (# 16-20204; reference #227030).

Periodontal Ligament Cell Harvesting and Cell Culture

Human primary periodontal ligament (PDL) cells were grown as explants from periodontal ligament tissues that were harvested from extracted teeth as previously described (Kapila, Kapila et al. 1996). All cells were cultured in alpha minimal essential medium (MEM-α) supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin-streptomycin, and 1% amphotericin B (Gibco, USA). All cells were used between passages 3 and 7. Use of PDL cells for these studies was approved by the University of California San Francisco IRB. Two donor samples were pooled together per independent sample to eliminate variation for all experiments except shRNA experiments (only one donor per independent sample was used for these experiments).

Microbial Culturing of *T. denticola*

T. denticola ATCC 35405 (American Type Culture Collection; Manassas, VA) and *T. denticola* mutants (CF522, isogenic dentilisin-deficient strain) (Godovikova, Wang et al. 2010), MHE (isogenic Msp-deficient strain) (Fenno, Wong et al. 1997), were cultured at 37°C under anaerobic conditions in oral treponeme enrichment broth (OTEB; Anaerobe Systems, Morgan Hill, CA). Culture purity was monitored using Syto 9 bacterial DNA dye and visualized in a fluorescent microscope for spirochete morphology. *T. denticola* was washed with MEM-α cultured media, centrifuged, aspirated thrice, and finally resuspended in MEM-α to be used for experiments. Bacteria used for all experiments never exceeded 6 passages.

Purified Dentilisin

The dentilisin protease complex was purified from the detergent phase of the Triton X-114 extracts of *T. denticola* MHE by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a model 491 Prep Cell (Bio-Rad Laboratories, Richmond, CA) as described previously (Fenno, Hannam et al. 1998, Miao, Fenno et al. 2011). Dentilisin purity was assayed in silver stained SDS-PAGE (Miao, et al. 2011). Purifed dentilisin migrated as a 100 kDa complex that upon heating resolves to its individual protein components: PrtP, PrcA1 and PrcA2, as documented previously (Miao, et al. 2011)."

MOI Optimization

Multiplicity of infection (MOI) was optimized by performing a PDL cell viability assay with *T. denticola* at different MOIs (10, 50, and 100). This viability assay (Calcein AM; Thermo Fisher Scientific) was used according to manufacturer's instructions. Cytotoxicity was encountered at an MOI of 500 (data not shown). In previous publications (Miao, Godovikova et al. 2014, Ateia, Sutthiboonyapan et al. 2018), a *T. denticola* MOI of 50 rendered a strong cellular response in PDL cells without inducing cytotoxicity. Based on these observations, an MOI of 50 was considered optimal for studying the cellular response of PDL cells to *T. denticola* treatment.

Treatment of PDL cells with *T. denticola*

PDL cells were plated in 4-well chamber slides (Invitrogen), 8-well chamber slides (Invitrogen), or 60mm culture plates (Falcon) overnight at a density of 3x104, 1.5x104 or 1.0x106 cells/well, respectively. Cells were then challenged with *T. denticola* at an optimized MOI of 50 for 2 h or left unchallenged as controls. Post-challenge, cells were washed three times with phosphate buffered saline (PBS, Gibco, USA) and incubated in MEM-α media (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% amphotericin B (Gibco, USA), and 1% penicillin-streptomycin for 24 h.

General Chemicals

All general chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise noted.

Imaging Stress Fibers via Immunofluorescence Staining

Human PDL cells were grown to confluency and passaged in sterile 4-well glass bottom plates (627870; Gibco, USA) 1.5x10-5 cells per well. Cells were initially challenged using the aforementioned protocol. Following incubation, cells were fixed with 4% paraformaldehyde for 15 mins at room temperature, washed with PBS, incubated with 0.2M glycine for 20 mins, washed with PBS, and permeablized with 0.2% Triton X-100 for 2 mins. Then cells were washed with PBS, incubated with blocking buffer (10% fetal bovine serum in PBS) for 5 mins, washed with PBS, incubated with Hoechst nuclear probe (Sigma 33342; 1:3000) and SiR-Actin (Cytoskeleton Inc; probe for F-actin 1:5000) for 40 mins at 37°C, washed with PBS, and mounted with Faramount mounting media (Dako). All images were captured with a Leica TCS SP8 Confocal Laser Scanning Microscope built on a Leica DMi8 inverted confocal laser scanning microscope (NA 0.95, 506375, Leica). Leica Application Suite X (LASX) imaging software was used for image capture. Images were taken in the xy format at 63x or 20x magnification in oil immersion. LASX software preset fluorescence filters were used for the following: Hoechst (nuclear stain; ThermoFisher Scientific) and SiR-Actin Alexa Fluor 647 (F-actin stain; Cytoskeleton Inc.) The relative optical intensity (ROI) was measured using imaging analysis software (FIJI, Image J, National Institutes of Health).

Live Imaging Immunofluorescence Staining Protocol

Human PDL cells (PDL) were grown to confluency and passaged in sterile 4-well glass bottom plates (627870; Gibco, USA) 1.5x10-5 cells per well. Before challenge, cells were incubated with SiR-Actin (1:5000 dilution) overnight, and Hoechst nuclear probe (Sigma 33342; 1:3000) for 30 mins. Additionally, wildtype (WT) *T. denticola* cells were incubated 20 mins before challenge with Syto 9 dye (1:500), vortexed and washed with MEM-α media twice before challenge. Syto 9 staining has been used previously in bacterial culture assays with *T. denticola* and the stain did not interfere with the functioning or viability of the pathogen (Yamada, Ikegami et al. 2005). PDL cells were challenged using the

aforementioned protocol. PDL cells treated with WT *T. denticola* (50 MOI) or control media were imaged at 15-minute intervals from 0 to 24h at set landmarks. All images were captured with a Zeiss scanning confocal microscope at 20x magnification with fluorescence filters for Hoechst, Alexa Flour 488 filter (Syto 9 dye for DNA; ThermoFisher Scientific) and Alexa Fluor 647 filter (SiR-Actin probe). The relative optical intensity (ROI) was measured using imaging analysis software (FIJI, Image J, National Institutes of Health).

Western Blot Analysis

PDL cells were plated in 60 mm tissue culture plates at 1.5x10-6 cells/plate overnight. Cells were then challenged with wildtype *T. denticola* at an MOI of 50 for 2 h or left unchallenged as controls. Post-challenge, cells were washed three times with PBS and incubated in MEM-α medium for 24hrs. Cells were mechanically harvested with a cell-scraper, pelleted under centrifugation, washed with PBS, and lysed using radioimmunoprecipitation assay (RIPA) buffer. Lysates were then electrophoretically resolved on pre-made 4-12% bis-tris polyacrylamide gels (Invitrogen) and transferred onto immobilon-P PVDF transfer membranes (EMD Millipore) for western blotting analysis to evaluate changes in actin monomers (β-actin and γ-actin). Primary antibodies included: β-actin (Abcam; ab8227; RRID:AB_2305186), γ-actin (Santa Cruz; Cat# sc-65635, RRID:AB_1120816), and glyceraldehye 3-phosphate dehydrogenase (GAPDH) (Santa Cruz; sc-32233, RRID:AB_627679). Supersignal West Pico Plus Chemiluminescent substrate solution (Thermo Scientific) was used to develop protein bands. Densitometry was performed using FIJI imaging analysis software.

RNA Sequencing

Three replicates of primary human periodontal ligament cells (PDL) were derived from three subjects. Cells were challenged with *T. denticola* treatment at a multiplicity of infection of 50 whereas control cells were challenged with MEM-α media. At the end of the 24 h incubation period, samples were pelleted,

frozen and sent to Novogene Corporation Inc. for RNA extraction, sequencing, and analysis. All samples passed quality control with a Q30 above 80% and sequencing was performed using the Illumina Platform PE150. The RNA-Sequencing data being presented is a subset of genes from the overall analysis. 165 genes were identified from the gene ontology pathways derived from the RNA sequencing data. Biological processes were identified from the top significant differentially regulated processes seen in the 5 h and 24 h gene ontology graphs (Supporting Information, Figure 2; Figure 4). Pathways identified were titled regulation of actin cytoskeleton organization, positive regulation of cell projection organization, regulation of actin filament organization, ras protein signal transduction, regulation of small GTPase meditated signal transduction, extracellular matrix component, and extracellular matrix organization. Based on these pathways, the top genes were identified and their adjusted p-value and differential expression was plotted as a volcano plot (Figure 4). All genes used in the volcano plot came from the 24 h challenge data sets within the pathways specified above. RASA4 was identified as one of the top genes upregulated upon the 24 h *T. denticola* challenge.

Detachment Assay

Cells were challenged using the aforementioned protocol above. Following incubation, cells were vigorously washed with PBS three times. Vigorous washings included full force application of PBS with a 1mL pipette directly on cells and aspiration on high. This is different from normal culture methods; normally, PBS is added slowly on the side of the chamber/plate wall and aspirated on low. Cells were then fixed with 4% paraformaldehyde for 15 mins at room temperature, washed with PBS, incubated with 0.2M Glycine for 20 mins, washed with PBS, permeablized with 0.2% Triton X-100 for 2 mins, washed with PBS, incubated with blocking buffer (10% fetal bovine serum in PBS; Gibco, USA) for 5 mins, washed with PBS (Gibco, USA), incubated with Hoechst (1:3000) and mounted with fluorescence mounting media (Faramount, Dako). Remaining cells were counted from 5 locations per well and cell number/location were averaged to constitute one experimental group. Mean cell count per experimental group was graphed as a ratio; control samples to wildtype *T. denticola* 50 MOI. Three samples were used per control

and wildtype *T. denticola* groups. The images are from one representative experiment. The graph represents results from three separate experiments. Data were compared using a Student's t-test. All images were captured with a Zeiss confocal microscope at 63x and 20x magnification with Hoechst and Alexa 647 (SiR-Actin) fluorescence filters, then cells were counted using imaging analysis software (FIJI).

Gelatin Zymography

Gelatin zymography was conducted as previously described (Miao, Fenno et al. 2011). Protein concentrations were calculated for each sample using the Pierce BSA Protein Assay kit according to manufacturer's instructions (Cat # 23225, Thermo Scientific). Equal protein for each sample was mixed with 4x sample buffer (0.25 M Tris base, 0.8% SDS, 40% glycerol, and 0.05% bromophenol blue), loaded into each well, and subjected to SDS-PAGE at 4°C on 10% gels containing 2 mg/ml gelatin. After electrophoresis, SDS was removed from the gels by washing them in renaturing buffer (2.5% Triton X-100, 50 mM Tris base) twice for 30 min then gels were placed in developing buffer (50 mM Tris base, pH 8, 10 mM CaCl2, and 0.02% NaN3) for 30 mins. Developing buffer was changed and gels were incubated at 37°C for 16 h then stained with 0.05% Coomassie blue for 30 mins, and de-stained in 10% acetic acid and 40% methanol until clear bands in a blue background were visible.

Quantitative RT-PCR (qRT-PCR)

RNA was isolated from PDL cells using the RNeasy Mini kit (Qiagen, Valencia, CA), reverse transcribed to cDNA using SuperScript Vilo cDNA Synthesis Kit (Invitrogen; Cat# 11754-050) and amplified by qRT-PCR using gene-specific primers for RASA4 (F: 5′-AGCGCAGCTCGCTGTACATC- 3'; R: 5′-GGCAGGTGCACTTGGTACTC- 3'), β-ACTIN (F: 5′-TGTTAGCGAGGGAGCAGTGG-3′; R: 5′-CCCATCGCCAAAACTCTTCA-3′), and GAPDH (F: 5′-TTGAGGTCAATGAAGGGGTC-3′; R: 5′-GAAGGTGAAGGTCGGAGTCA-3′) (Jin, Wang et al. 2007). Cycle threshold values of the genes of interest and the quantitative gene expression levels normalized to GAPDH for each experimental sample were determined and compared with that of unchallenged control samples.

Transduction of PDL cultures with RASA4 shRNA

Short hairpin RNA (shRNA) lentiviral particles specific for RASA4 (Santa Cruz Biotechnology; sc-89880-V) was used to inhibit RASA4 expression in PDL cells, according to manufacturer's instructions. Briefly, PDL cells were incubated with polybrene reagent (EMD Millipore, USA) at a final concentration of 5ug/ml and RASA4 shRNA or scrambled shRNA (control) lentiviral particles (sc-108064) for 10 h. After infection, PDL cell media was changed with normal supplemented MEM-α media overnight. Lentiviral particles used contain a puromycin resistance gene used for selection. Selection of clones with successfully intake of knockdown constructs were identified by treating infected hPDL cells with puromycin (Calbiochem, USA) at a final concentration of 5 µg/mL until single cell colonies remained. Experiment ended by washing with phosphate buffered saline (PBS, Gibco, USA) and replaced with normal growth medium (MEM-α media supplemented with 10% fetal bovine serum (Gibco, USA), 1% amphotericin B (Gibco, USA), and 1% penicillin-streptomycin). Cells were passaged once and validated via RT-qPCR before used for experiments

Actin Dynamics Experiments

Actin polymerization for all experiments was stimulated by Jasplakinolide treatment (Jasp) of PDL cells at a concentration of 500nM for 1 h in MEM-α alone media prior to *T. denticola* challenge and throughout *T. denticola* challenge as previously described. Control samples were challenged with MEM-α alone media with no bacterial cells. These cells were labelled as Jasplakinolide alone. Actin depolymerization for all experiments was stimulated by Latrunculin B treatment (Lat B) of PDL cells at a concentration of 500nM for 1 h in MEM-α alone media prior to *T. denticola* challenge and throughout *T. denticola* challenge as previously described. Control samples were challenged with MEM-α alone media with no bacterial cells. These cells were labelled as Latrunculin B alone. Post-challenge, media was removed, washed

with PBS thrice, and replaced with MEM-α only media. Media was collected after the 24 h incubation for gelatin zymography. Cells were then incubated for 24h, then processed for RNA extraction for qRT-PCR.

Statistical Analysis

All data used in this manuscript was analyzed using the statistical software GraphPad Prism version 8.4.3 (La Jolla California USA). Results were evaluated by The Student's t test when comparing two groups. A one-way analysis of variance (ANOVA) was used when comparing three or more groups. ANOVA Turkey's multiple comparison test was used to distinguishing differences of two groups within experiments with three or more groups. The Two-way ANOVA test was used compare more than three groups, while comparing differences between shRNA scrambled and shRNA RASA4 knockdown samples. P value of ≤ .05 was considered statistically significant. Annotation within figure legends identify *= $p \leq .05$, **= $p \leq .01$, ***= $p \leq .001$

Chapter 2.6: FIGURES

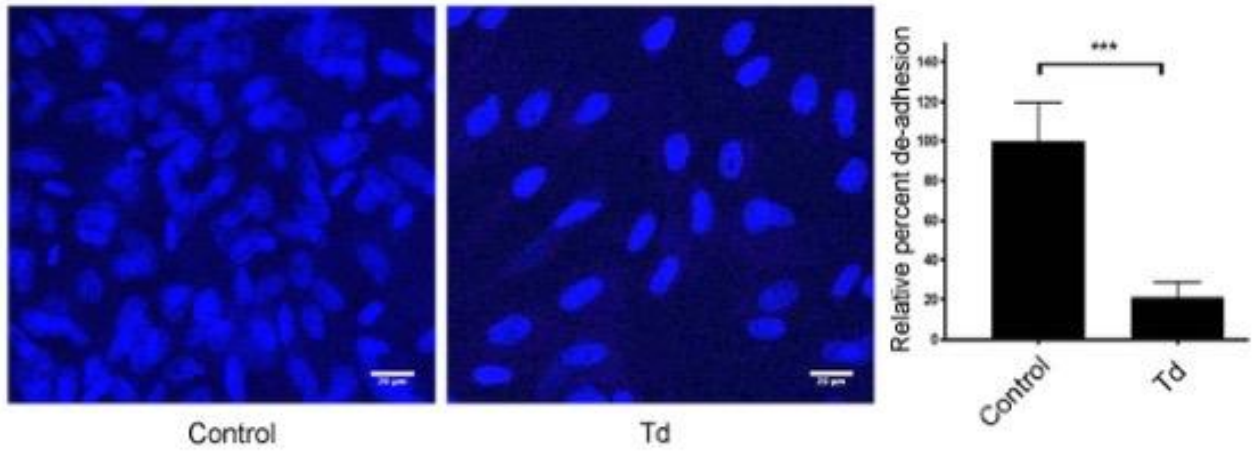


Figure 2.3.1 PDL cell attachment and contractility is impaired by T. denticola challenge.

PDL cells were unchallenged (control) or challenged with T. denticola (50 MOI) for 24 h and processed for immunofluorescent staining with Hoechst (nucleus, blue), and imaged with Confocal Microscopy. Representative phase contrast images are shown, and graphs show the percent change in de-adhesion after 24 h. Data represent mean ± SD from three independent experiments. Data were compared using Student's t-test. *** = $p<0.001$; N.S.= not significant

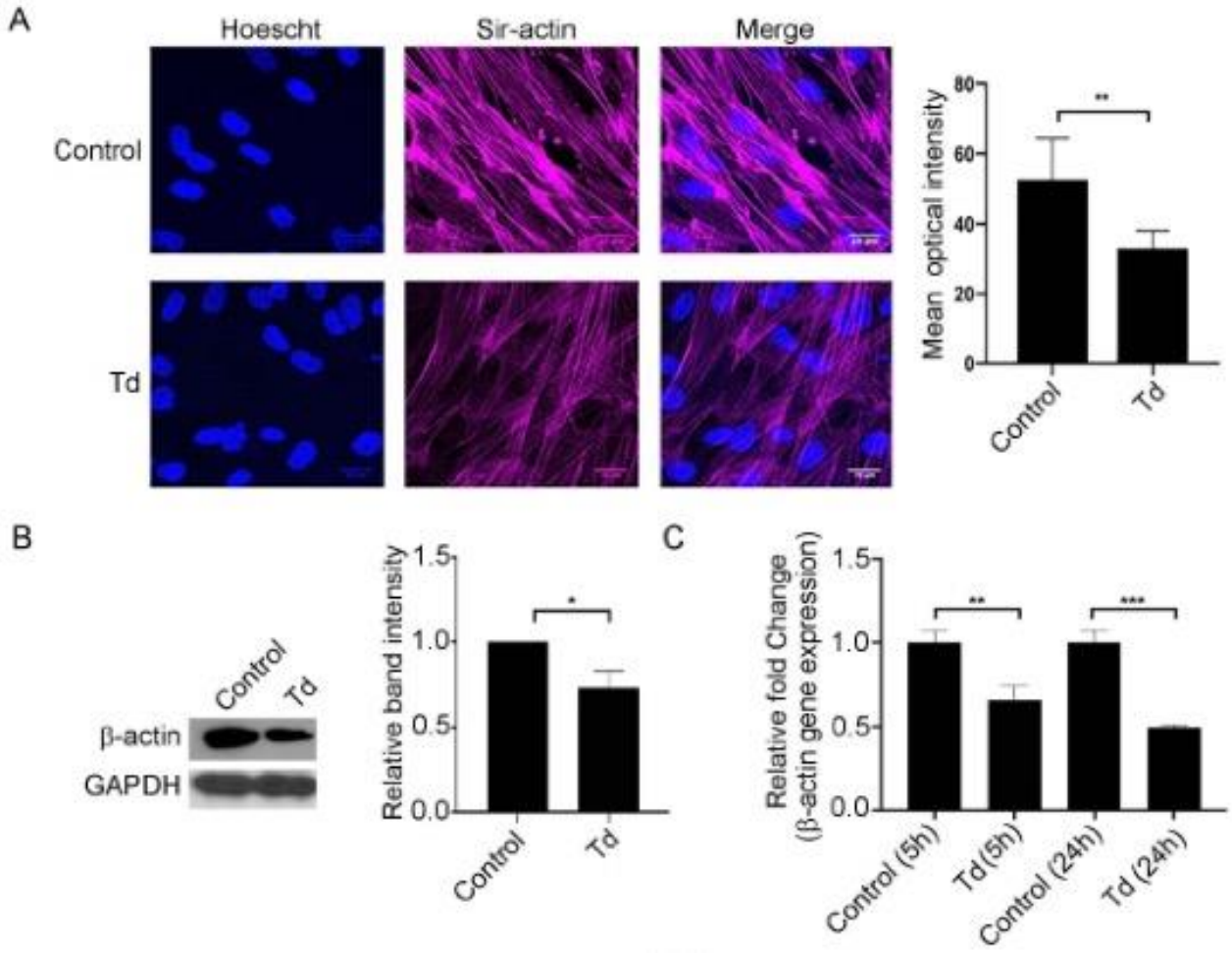


Figure 2.3.2 *T. denticola* challenge decreases stress fibers and β-actin protein monomer abundance in PDL cells. Panel A: (Left) PDL cells were unchallenged (control) or challenged with *T. denticola* (50 MOI) for 24 h and processed for immunofluorescent staining with Hoechst (nucleus, blue) and SiR-Actin probe, and imaged using confocal microscopy. (Right) Relative optical intensities were calculated from three independent experiments. Panel B: (Left) Representative immunoblot showing β-actin levels in PDL cells challenged with control medium or media containing *T. denticola* (50 MOI) for 24 h. (Right), Densitometry analysis of three independent experiments was performed using ImageJ software, and the ratio of control samples to *T. denticola* was calculated. Data represent mean ± SD from three independent experiments. Data were compared using Student's t-test. * = $p<0.05$; ** = $p<0.01$. Panel C: β-actin gene expression in PDL cells challenged with control medium or media containing *T. denticola* (50 MOI) for 5h or 24 h by RNA-Seq. The graph shows the fold changes on a log2 scale. Data were compared using Student's t-test. ** = $p<0.01$; *** = $p<0.001$.

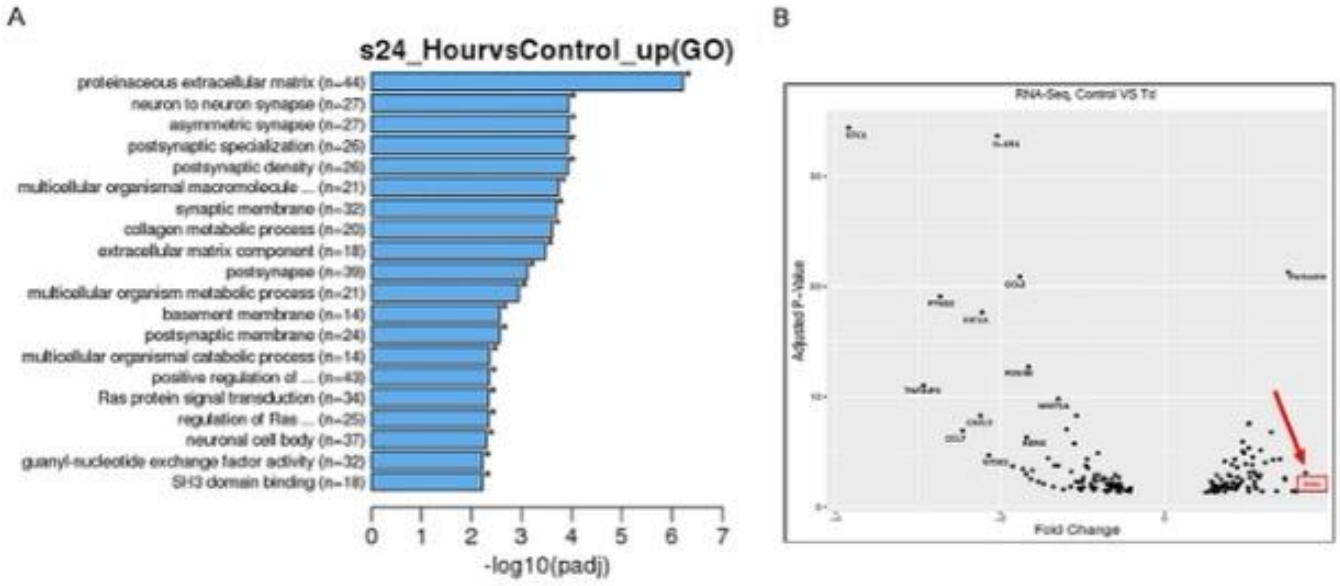


Figure 2.3.3. Gene pathways and volcano plot shows RASA4 as a top upregulated gene in *T. denticola* challenged PDL cells. PDL cells were unchallenged or challenged with *T. denticola* (50 MOI) for 24 h and differential gene expression analysis was performed using RNA-Seq. 165 genes were identified from the gene ontology pathways derived from the RNA sequencing data. Biological processes were identified from the top significant processes that were differentially regulated in the gene ontology enrichment analysis from 5 h and 24 h gene ontology graphs (Supporting Information, Figure 2; Figure 3A). Pathways identified were titled regulation of actin filament organization, positive regulation of cell projection organization, regulation of actin filament organization, ras protein signal transduction, regulation of small GTPase meditated signal transduction, extracellular matrix component, and extracellular matrix organization. Based on these pathways, the top genes were identified and their adjusted p-value and differential expression was plotted as a volcano plot (Figure 3B). All genes used in the volcano plot came from the 24 h challenge data sets within the pathways specified above. RASA4 was identified as one of the top genes upregulated upon 24 h *T. denticola* challenge.

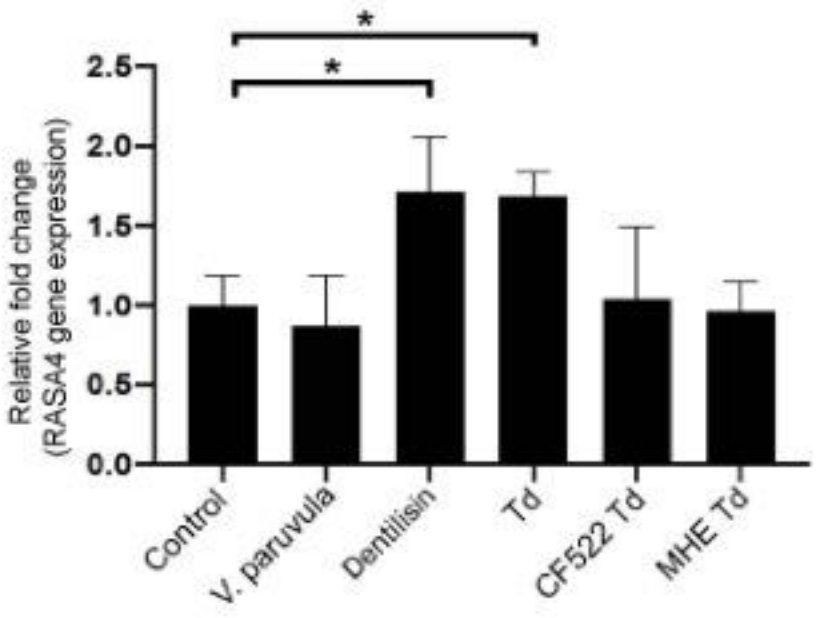


Figure 2.3.4. Purified dentilisin and *T. denticola* upregulate RASA4 gene expression in PDL cells. Quantitative RT-PCR analysis of RASA4 relative fold change in PDL cells challenged with V. parvula, wild type *T. denticola*, *T. denticola* dentilisin mutant CF522, *T. denticola* MHE or treated with purified dentilisin for 24 h. Data represent mean ± SD from three independent experiments. Data were compared using One-way ANOVA Tukey's Multiple comparisons test. *= $p<0.05$

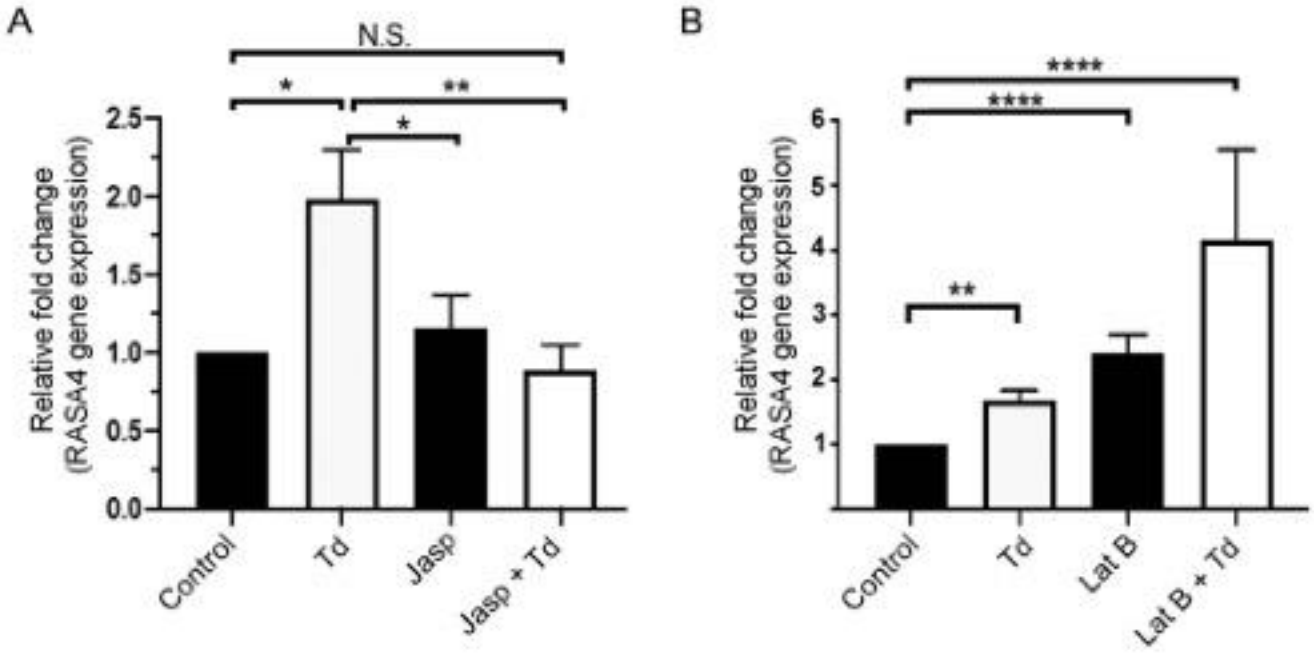


Figure 2.3.5. Jasplakinolide inhibited *T. denticola*-induced RASA4 gene expression, whereas RASA4 gene expression is upregulated in Latrunculin B pretreated and *T. denticola* challenged PDL cells. Quantitative RT-PCR analysis of RASA4 relative fold change in the gene expression of PDL cells treated with Jasplakinolide (Jasp; panel A) or Latrunculin B (Lat B; panel B) and challenged with *T. denticola* (50 MOI). Data represent mean ± SD from three independent experiments. Data were compared using Two-way ANOVA Tukey's Multiple comparisons test. * = $p<0.05$; ** = $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$; N.S.= not significant.

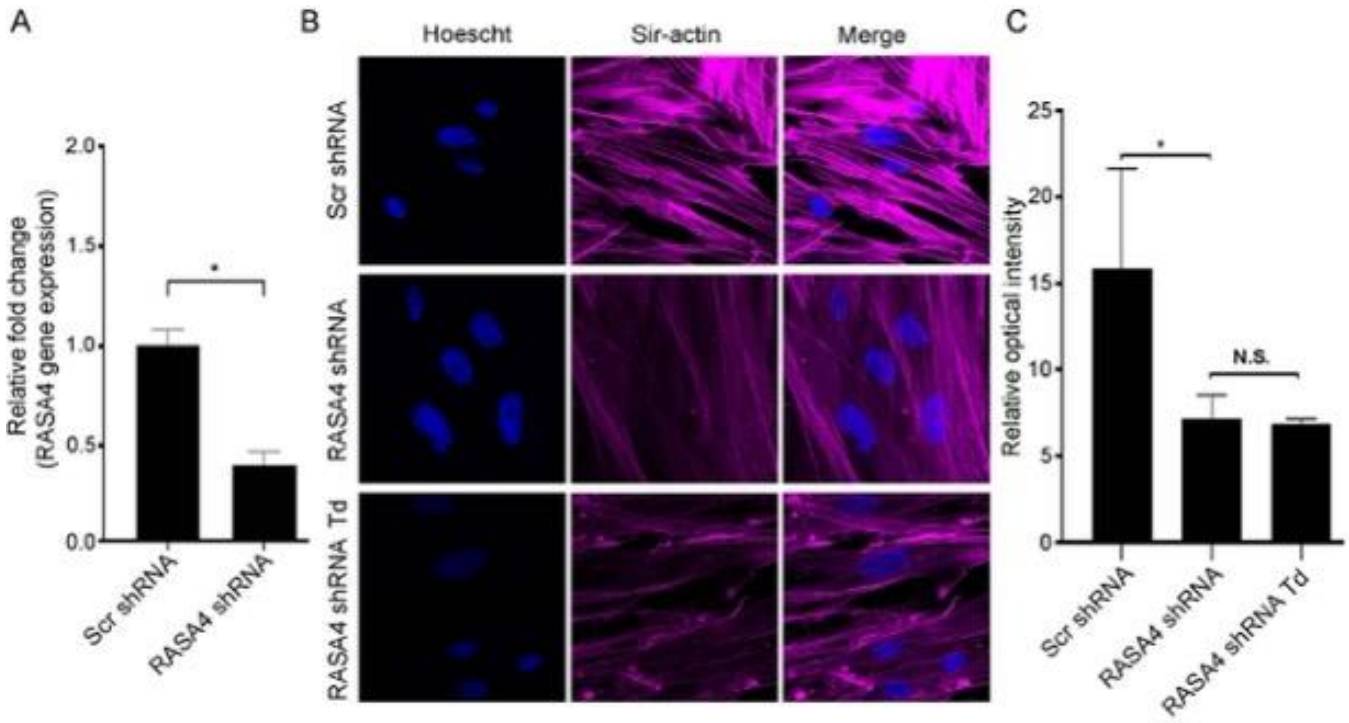


Figure 2.3.6. RASA4 gene expression is required for *T. denticola*-mediated actin stress fiber dysfunction. Panel A: Quantitative RT-PCR analysis of RASA4 relative fold change in PDL cells infected with scramble shRNA or RASA4 shRNA. Panel B: Representative confocal images and (panel C) relative optical intensity of stress fiber intensity in scramble shRNA, RASA4 shRNA and RASA4shRNA cells challenged cells with T. denticola (50 MOI) for 24 h and stained with SiR-Actin dye (1:5000). Data represent mean ± SD from three independent experiments. Data in panel A was analyzed using an Unpaired t test. Data in panel C was analyzed using One-way ANOVA Tukey's Multiple comparisons test. *= $p<0.05$.

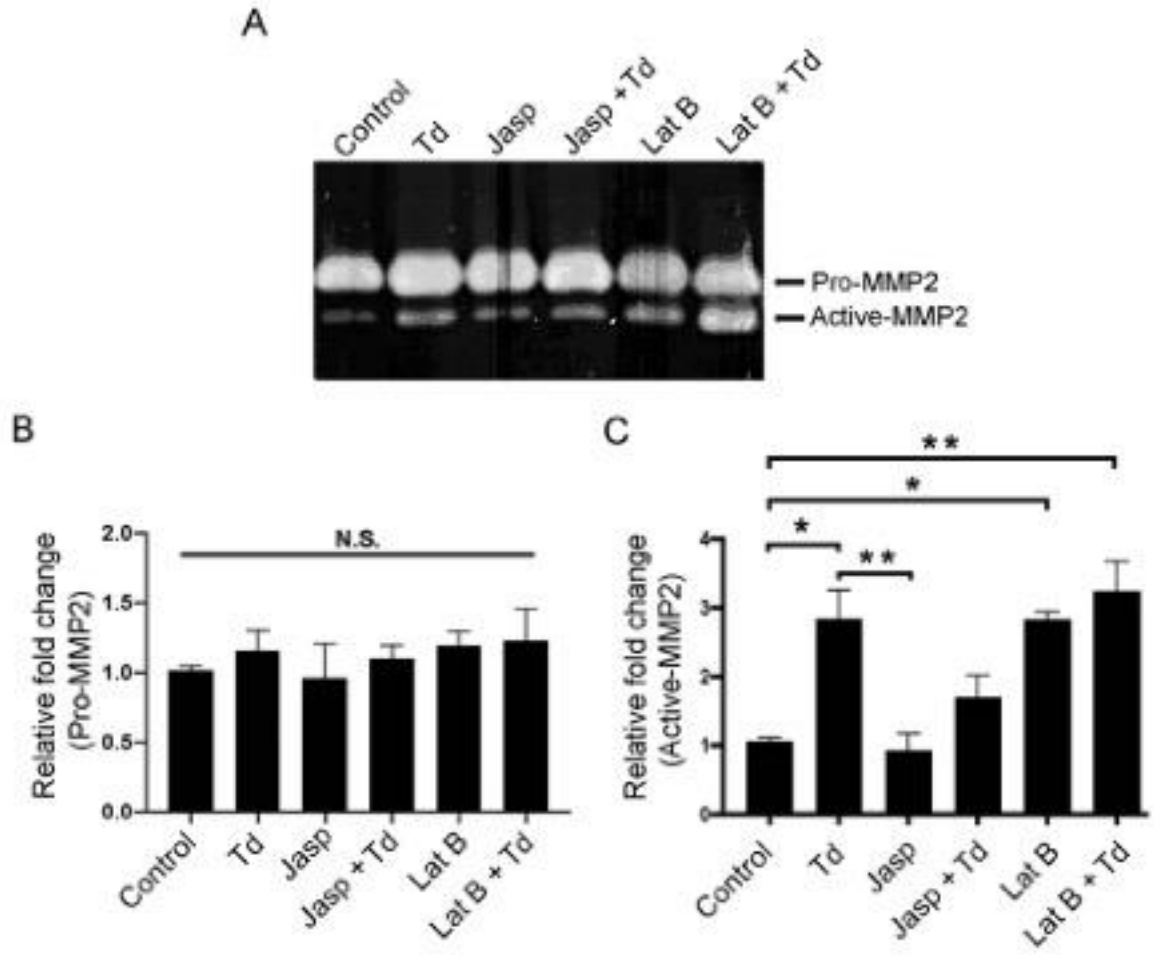


Figure 2.3.7. Actin depolymerization through *T. denticola* challenge and Latrunculin B pretreatment increases MMP-2 enzymatic activity. Actin polymerizing agent Jasplakinolide (Jasp) and actin depolymerizing agent Latrunculin B (Lat B) were used to pretreat PDL cells for one h. Cells were then unchallenged or challenged with *T. denticola* (50 MOI) for 24 h. Cultured media was collected and gelatin zymography was used to measure MMP-2 activity. A representative Gelatin zymogram is shown (7A) and densitometry analysis of pro-MMP-2 (7B) and active-MMP-2 (7C). Data represent mean ± SD from three independent experiments. Data were compared using One-way ANOVA Tukey's Multiple comparisons test. *. = $p<0.05$; ** = $p<0.01$; N.S.= not significant

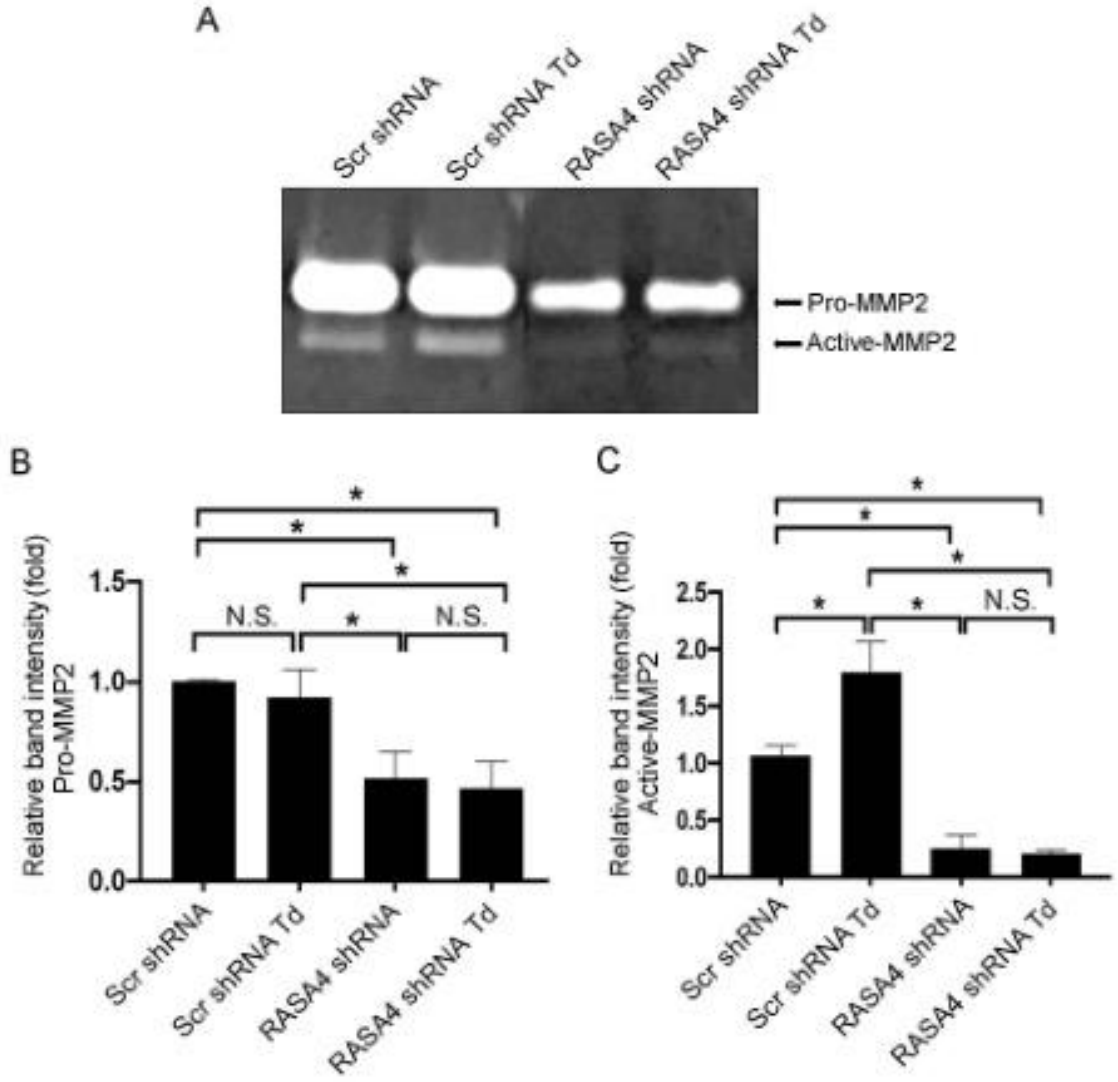
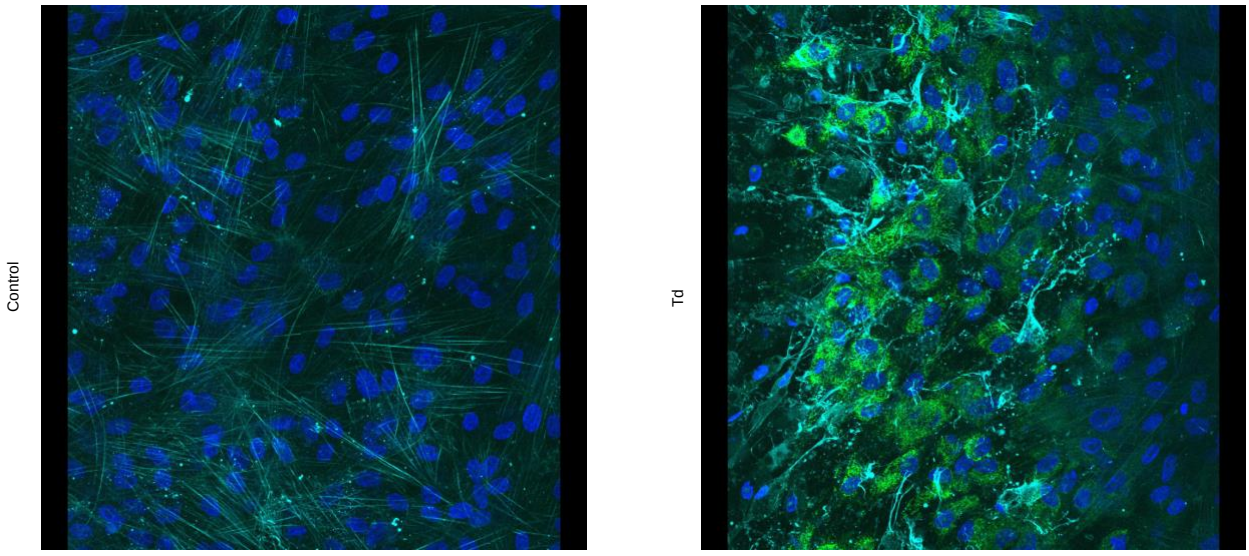
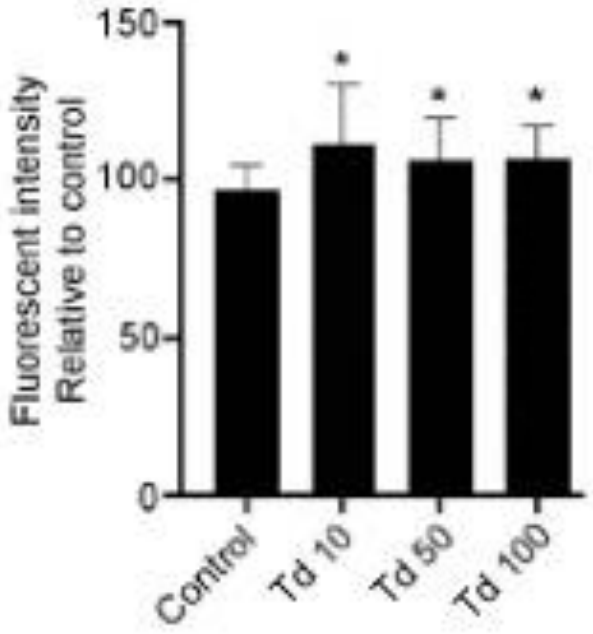


Figure 2.3.8. RASA4 gene expression is required for *T. denticola*-mediated enhancement of MMP-2 activity. Pro-MMP-2 and active-MMP-2 in scramble shRNA and RASA4shRNA cells challenged with *T. denticola* (50 MOI) for 24 h. Panel A: a representative zymogram. Panels B (Pro-Mmp-2) and C (active Mmp-2) show mean ± SD from three independent experiments. Data was compared using Two-way ANOVA Tukey's Multiple comparisons test. *= $p<0.05$; N.S.= not significant

Chapter 2.7: SUPPLEMENTAL FIGURES

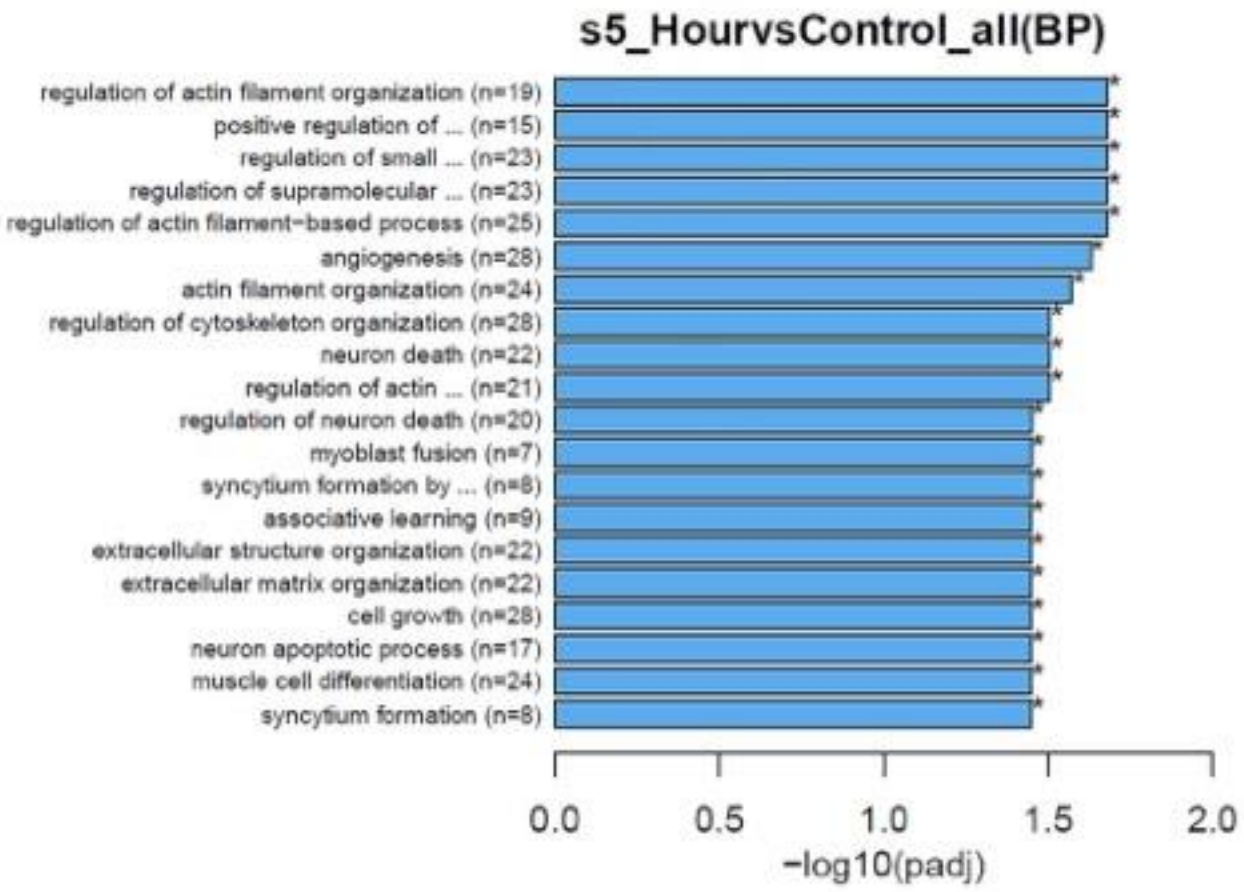


Supplemental Figure: 2.7.1 *T. denticola* interaction negatively influences PDL cell actin filament functioning. Approximately 1.0x10^5 PDL cells per well were plated in a 4-well glass bottom culture plate. SiR-Actin dye (1:5000) and Hoechst (1:3000) stain were added to each well 4 hours before imaging. *T. denticola* was pre-incubated in a 15mL falcon tube with Syto9 (1:500) fluorescent dye for 20 mins. Bacteria were washed and centrifuged twice to remove unbound Syto9 and cells were reconstituted with MEM-α media. Syto9-labelled *T. denticola* (50 MOI) was added to plated PDL cells. Control cells contained no *T. denticola*. Culture plates were imaged in a sterile chamber containing 5% CO2 and connected to a scanning laser confocal microscope. Images were set to image every 15 minutes for 24 hours. Maximum projections (representative image) and each individual image were analyzed in FIJI software. Hoechst, SiR-Actin, and Syto9 were assigned blue, cyan, and green filters, respectively. Images were combined to create the imaging video. Link to videos are under the data availability statement.



Supplemental Figure 2

Supporting Information Figure 2.7.2 *T. denticola* interaction does not affect viability of PDL Cells. PDL cells were plated in 96 well plate (approximately 3x 10^4 cell per well) unchallenged (control) or challenged with *T. denticola* (10, 50, or 100 MOI) for 24 h and Calcein AM assays were performed according to manufacturer's instructions. Data represent mean ± SD from three independent experiments containing 10 wells per sample. Data was compared using One-way ANOVA Tukey's Multiple comparisons test. *= $p<0.05$; N.S.= not significant



Supplemental Figure 2.7.3 *T. denticola* interaction reflects an effect on actin and cytoskeletal biological processes. Gene ontology enrichment was performed from RNAseq data previously described. Gene ontology for PDL cells challenged for 5 h with *T. denticola* versus control (unchallenged PDL cells) showed a significant differential expression. Top differentially expressed biological processes include: regulation of actin filament organization and actin filament-based process, actin filament organization, regulation of cytoskeletal organization, regulation of actin organization, extracellular matrix organization.

Chapter 2.8: REFERENCES

1. Amin, M., A. C. Ho, J. Y. Lin, A. P. Batista da Silva, M. Glogauer and R. P. Ellen (2004). "Induction of *de novo* subcortical actin filament assembly by *Treponema denticola* major outer sheath protein." Infect Immun **72**(6): 3650-3654.
2. Armitage, G. C. (2004). "Periodontal diagnoses and classification of periodontal diseases." Periodontol 2000 **34**: 9-21.
3. Ateia, I. M., P. Sutthiboonyapan, P. Kamarajan, T. Jin, V. Godovikova, Y. L. Kapila and J. C. Fenno (2018). "*Treponema denticola* increases MMP-2 expression and activation in the periodontium via reversible DNA and histone modifications." Cell Microbiol **20**(4).
4. Baehni, P. C., M. Song, C. A. McCulloch and R. P. Ellen (1992). "*Treponema denticola* induces actin rearrangement and detachment of human gingival fibroblasts." Infect Immun **60**(8): 3360-3368.
5. Bildyug, N. (2016). "Matrix metalloproteinases: an emerging role in regulation of actin microfilament system." Biomol Concepts **7**(5-6): 321-329.
6. Chintala, S. K., R. Sawaya, B. B. Aggarwal, S. Majumder, D. K. Giri, A. P. Kyritsis, Z. L. Gokaslan and J. S. Rao (1998). "Induction of matrix metalloproteinase-9 requires a polymerized actin cytoskeleton in human malignant glioma cells." J Biol Chem **273**(22): 13545-13551.
7. Choi, C. and D. M. Helfman (2014). "The Ras-ERK pathway modulates cytoskeleton organization, cell motility and lung metastasis signature genes in MDA-MB-231 LM2." Oncogene **33**(28): 3668-3676.
8. de Araujo, R. M., Y. Oba, S. Kuroda, E. Tanaka and K. Moriyama (2014). "RhoE regulates actin cytoskeleton organization in human periodontal ligament cells under mechanical stress." Arch Oral Biol **59**(2): 187-192.

9. Fenno, J. C., P. M. Hannam, W. K. Leung, M. Tamura, V. J. Uitto and B. C. McBride (1998). "Cytopathic effects of the major surface protein and the chymotrypsinlike protease of *Treponema denticola*." Infect Immun **66**(5): 1869-1877.
10. Fenno, J. C., G. W. Wong, P. M. Hannam, K. H. Müller, W. K. Leung and B. C. McBride (1997). "Conservation of *msp*, the gene encoding the major outer membrane protein of oral *Treponema* spp." J Bacteriol **179**(4): 1082-1089.
11. Fernández, A., A. M. Cárdenas, J. Astorga, P. Veloso, A. Alvarado, P. Merino, D. Pino, D. Reyes-Court and M. Hernández (2019). "Expression of Toll-like receptors 2 and 4 and its association with matrix metalloproteinases in symptomatic and asymptomatic apical periodontitis." Clin Oral Investig **23**(12): 4205-4212.
12. Godovikova, V., H. T. Wang, M. P. Goetting-Minesky, Y. Ning, R. F. Capone, C. K. Slater and J. C. Fenno (2010). "*Treponema denticola* PrcB is required for expression and activity of the PrcA-PrtP (dentilisin) complex." J Bacteriol **192**(13): 3337-3344.
13. Gursoy, U. K., E. Könönen and V. J. Uitto (2008). "Stimulation of epithelial cell matrix metalloproteinase (MMP-2, -9, -13) and interleukin-8 secretion by fusobacteria." Oral Microbiol Immunol **23**(5): 432-434.
14. Gutiérrez-Venegas, G., P. Kawasaki-Cárdenas, C. P. Garcés, P. Román-Alvárez, C. Barajas-Torres and L. A. Contreras-Marmolejo (2007). "*Actinobacillus actinomycetemcomitans* adheres to human gingival fibroblasts and modifies cytoskeletal organization." Cell Biol Int **31**(9): 1063-1068.
15. Hasegawa, Y., G. D. Tribble, H. V. Baker, J. J. Mans, M. Handfield and R. J. Lamont (2008). "Role of *Porphyromonas gingivalis* SerB in gingival epithelial cell cytoskeletal remodeling and cytokine production." Infect Immun **76**(6): 2420-2427.
16. Hassell, T. M., P. Baehni, E. L. Harris, C. Walker, G. Gabbiani and A. Geinoz (1997). "Evidence for genetic control of changes in f-actin polymerization caused by pathogenic microorganisms:

in vitro assessment using gingival fibroblasts from human twins." J Periodontal Res **32**(1 Pt 2): 90-98.

17. Humphrey, J. D., E. R. Dufresne and M. A. Schwartz (2014). "Mechanotransduction and extracellular matrix homeostasis." Nat Rev Mol Cell Biol **15**(12): 802-812.
18. Jang, A. T., L. Chen, A. R. Shimotake, W. Landis, V. Altoe, S. Aloni, M. Ryder and S. P. Ho (2018). "A Force on the Crown and Tug of War in the Periodontal Complex." J Dent Res **97**(3): 241-250.
19. Jin, H., X. Wang, J. Ying, A. H. Wong, Y. Cui, G. Srivastava, Z. Y. Shen, E. M. Li, Q. Zhang, J. Jin, S. Kupzig, A. T. Chan, P. J. Cullen and Q. Tao (2007). "Epigenetic silencing of a Ca(2+)-regulated Ras GTPase-activating protein RASAL defines a new mechanism of Ras activation in human cancers." Proc Natl Acad Sci U S A **104**(30): 12353-12358.
20. Jobin, M. C., I. Virdee, C. A. McCulloch and R. P. Ellen (2007). "Activation of MAPK in fibroblasts by *Treponema denticola* major outer sheath protein." Biochem Biophys Res Commun **356**(1): 213-218.
21. Kang, Y. G., J. H. Nam, K. H. Kim and K. S. Lee (2010). "FAK pathway regulates PGE(2) production in compressed periodontal ligament cells." J Dent Res **89**(12): 1444-1449.
22. Kanzaki, H., M. Chiba, Y. Shimizu and H. Mitani (2002). "Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis." J Bone Miner Res **17**(2): 210-220.
23. Kapila, Y. L., S. Kapila and P. W. Johnson (1996). "Fibronectin and fibronectin fragments modulate the expression of proteinases and proteinase inhibitors in human periodontal ligament cells." Matrix Biol **15**(4): 251-261.
24. Kapoor, P. and X. Shen (2014). "Mechanisms of nuclear actin in chromatin-remodeling complexes." Trends Cell Biol **24**(4): 238-246.

25. Kinane, J. A., M. R. Benakanakere, J. Zhao, K. B. Hosur and D. F. Kinane (2012). "*Porphyromonas gingivalis* influences actin degradation within epithelial cells during invasion and apoptosis." Cell Microbiol **14**(7): 1085-1096.
26. Ko, K. S., M. Glogauer, C. A. McCulloch and R. P. Ellen (1998). "*Treponema denticola* outer membrane inhibits calcium flux in gingival fibroblasts." Infect Immun **66**(2): 703-709.
27. Letra, A., G. Ghaneh, M. Zhao, H. Ray, Jr., C. F. Francisconi, G. P. Garlet and R. M. Silva (2013). "MMP-7 and TIMP-1, new targets in predicting poor wound healing in apical periodontitis." J Endod **39**(9): 1141-1146.
28. Li, L., T. T. Zeng, B. Z. Zhang, Y. Li, Y. H. Zhu and X. Y. Guan (2017). "Overexpression of HN1L promotes cell malignant proliferation in non-small cell lung cancer." Cancer Biol Ther **18**(11): 904-915.
29. Manokawinchoke, J., N. Limjeerajarus, C. Limjeerajarus, P. Sastravaha, V. Everts and P. Pavasant (2015). "Mechanical Force-induced TGFB1 Increases Expression of SOST/POSTN by hPDL Cells." J Dent Res **94**(7): 983-989.
30. Miao, D., J. C. Fenno, J. C. Timm, N. E. Joo and Y. L. Kapila (2011). "The *Treponema denticola* chymotrypsin-like protease dentilisin induces matrix metalloproteinase-2-dependent fibronectin fragmentation in periodontal ligament cells." Infect Immun **79**(2): 806-811.
31. Miao, D., V. Godovikova, X. Qian, S. Seshadrinathan, Y. L. Kapila and J. C. Fenno (2014). "*Treponema denticola* upregulates MMP-2 activation in periodontal ligament cells: interplay between epigenetics and periodontal infection." Arch Oral Biol **59**(10): 1056-1064.
32. Misu, S., M. Takebayashi and K. Miyamoto (2017). "Nuclear Actin in Development and Transcriptional Reprogramming." Front Genet **8**: 27.
33. Munshi, H. G., Y. I. Wu, E. V. Ariztia and M. S. Stack (2002). "Calcium regulation of matrix metalloproteinase-mediated migration in oral squamous cell carcinoma cells." J Biol Chem **277**(44): 41480-41488.

34. Osorio, C., F. Cavalla, A. Paula-Lima, G. Díaz-Araya, R. Vernal, P. Ahumada, J. Gamonal and M. Hernández (2015). "H2 O2 activates matrix metalloproteinases through the nuclear factor kappa B pathway and Ca(2+) signals in human periodontal fibroblasts." J Periodontal Res **50**(6): 798-806.

35. Pattamapun, K., S. Tiranathanagul, T. Yongchaitrakul, J. Kuwatanasuchat and P. Pavasant (2003). "Activation of MMP-2 by *Porphyromonas gingivalis* in human periodontal ligament cells." J Periodontal Res **38**(2): 115-121.

36. Ruby, J., M. Martin, M. J. Passineau, V. Godovikova, J. C. Fenno and H. Wu (2018). "Activation of the Innate Immune System by *Treponema denticola* Periplasmic Flagella through Toll-Like Receptor 2." Infect Immun **86**(1).

37. Sadhukhan, S., K. Sarkar, M. Taylor, F. Candotti and Y. M. Vyas (2014). "Nuclear role of WASp in gene transcription is uncoupled from its ARP2/3-dependent cytoplasmic role in actin polymerization." J Immunol **193**(1): 150-160.

38. Sanka, K., R. Maddala, D. L. Epstein and P. V. Rao (2007). "Influence of actin cytoskeletal integrity on matrix metalloproteinase-2 activation in cultured human trabecular meshwork cells." Invest Ophthalmol Vis Sci **48**(5): 2105-2114.

39. Serebryannyy, L. A., C. M. Cruz and P. de Lanerolle (2016). "A Role for Nuclear Actin in HDAC 1 and 2 Regulation." Sci Rep **6**: 28460.

40. Socransky, S. S., A. D. Haffajee, M. A. Cugini, C. Smith and R. L. Kent, Jr. (1998). "Microbial complexes in subgingival plaque." J Clin Periodontol **25**(2): 134-144.

41. Takeuchi, Y., M. Umeda, M. Sakamoto, Y. Benno, Y. Huang and I. Ishikawa (2001). "*Treponema socranskii*, *Treponema denticola*, and *Porphyromonas gingivalis* are associated with severity of periodontal tissue destruction." J Periodontol **72**(10): 1354-1363.

42. Tiranathanagul, S., K. Pattamapun, T. Yongchaitrakul and P. Pavasant (2004). "MMP-2 activation by *Actinobacillus actinomycetemcomitans* supernatant in human PDL cells was corresponded with reduction of TIMP-2." Oral Dis **10**(6): 383-388.

43. Tsuji, K., K. Uno, G. X. Zhang and M. Tamura (2004). "Periodontal ligament cells under intermittent tensile stress regulate mRNA expression of osteoprotegerin and tissue inhibitor of matrix metalloprotease-1 and -2." J Bone Miner Metab **22**(2): 94-103.

44. Visa, N. and P. Percipalle (2010). "Nuclear functions of actin." Cold Spring Harb Perspect Biol **2**(4): a000620.

45. Visser, M. B., A. Koh, M. Glogauer and R. P. Ellen (2011). "*Treponema denticola* Major Outer Sheath Protein Induces Actin Assembly at Free Barbed Ends by a PIP2-Dependent Uncapping Mechanism in Fibroblasts." PLoS One **6**(8): e23736.

46. Wang, Q., K. S. Ko, A. Kapus, C. A. McCulloch and R. P. Ellen (2001). "A spirochete surface protein uncouples store-operated calcium channels in fibroblasts: a novel cytotoxic mechanism." J Biol Chem **276**(25): 23056-23064.

47. Wang, W. Y., C. D. Davidson, D. Lin and B. M. Baker (2019). "Actomyosin contractility-dependent matrix stretch and recoil induces rapid cell migration." Nat Commun **10**(1): 1186.

48. Yamada, M., A. Ikegami and H. K. Kuramitsu (2005). "Synergistic biofilm formation by *Treponema denticola* and *Porphyromonas gingivalis*." FEMS Microbiol Lett **250**(2): 271-277.

49. Yang, P. F., M. Song, D. A. Grove and R. P. Ellen (1998). "Filamentous actin disruption and diminished inositol phosphate response in gingival fibroblasts caused by *Treponema denticola*." Infect Immun **66**(2): 696-702.

50. Yilmaz, O., P. A. Young, R. J. Lamont and G. E. Kenny (2003). "Gingival epithelial cell signalling and cytoskeletal responses to *Porphyromonas gingivalis* invasion." Microbiology (Reading) **149**(Pt 9): 2417-2426.

51. Zhang, J., J. Guo, I. Dzhagalov and Y. W. He (2005). "An essential function for the calcium-promoted Ras inactivator in Fcgamma receptor-mediated phagocytosis." Nat Immunol **6**(9): 911-919.

52. Zhang, W., J. Ju, T. Rigney and G. Tribble (2013). "Integrin alpha5beta1-fimbriae binding and actin rearrangement are essential for *Porphyromonas gingivalis* invasion of osteoblasts and subsequent activation of the JNK pathway." BMC Microbiol **13**: 5.
53. Zhao, K., W. Wang, O. J. Rando, Y. Xue, K. Swiderek, A. Kuo and G. R. Crabtree (1998). "Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling." Cell **95**(5): 625-636.

CHAPTER 3: Nisin-loaded Solid Lipid Nanoparticles (SLN-Nisin) abrogate *Treponema denticola* internalization and gene expression alterations in human periodontal ligament cells.

Chapter 3.1 ABSTRACT

Periodontal disease is a bacterially-driven chronic oral inflammatory disease that compromises the integrity of the tooth-supporting tissues, including the gingiva, periodontal ligament and alveolar bone. Given the bacterial nature of dental biofilms and the increased use of topical and systemic antibiotics to treat periodontal disease, the rise of multidrug-resistant microbes and the scarcity of new antibiotics are growing concerns. Therefore, novel approaches are needed to address these issues. One such approach is the use of nisin, an antimicrobial peptide, which can inhibit key periodontopathic bacteria, including *Treponema denticola*, and disrupt oral biofilms in *in vitro* and *in vivo* periodontal disease models without toxic effects or induction of resistance. In addition, nano-sized drug delivery systems can be used to optimize and/or enhance nisin capabilities. Therefore, the objective of this work was to test whether nisin-loaded SLN (SLN-Nisin) can treat *Treponema denticola*-infected human-derived periodontal ligament (hPDL) cells. We demonstrated that *T. denticola* is internalized by hPDL cells and colocalizes with early (Rab5-marked) and late (Rab7-marked) endosomes. Importantly, SLN-Nisin also internalizes and colocalizes with *T. denticola* inside of Rab7-marked late endosomes, thereby significantly reducing the *T. denticola* bacterial genetic material. Moreover, SLN-Nisin reverses 4 of the top altered gene expression by the bacterium back to control levels. To the best of our knowledge, this is the first demonstration of a nisin-based delivery system's ability to mitigate intracellular infection. These results indicate that SLN-nisin delivery systems are an innovative approach for improving antimicrobial peptides/bacteriocins for targeting intracellular pathogens, such as *Treponema denticola*-infected hPDL cells.

Chapter 3.2 INTRODUCTION

Periodontal disease is a bacterially-driven chronic oral inflammatory disease that compromises the integrity of the tooth-supporting tissues, including the gingiva, periodontal ligament and alveolar bone (1–3). The most recent National Health and Nutrition Examination Survey (NHANES) report (2009-2014) indicates that 42.2% of US adults 30 years or older have periodontitis, with almost a fifth of those considered severe periodontitis (4). Globally, it has been estimated that the disease treatment costs are over $400 billion US dollars per year and over $50 billion US dollars per year in loss of productivity (5). In addition, recent studies indicate a strong interplay between periodontal disease and other systemic diseases, such as oral cancer, diabetes, cardiovascular diseases and pre-term low birth weights (3,6–9), which could inflate these figures even further. Periodontal disease etiology has been extensively discussed in the literature, with the most accepted hypothesis today being the polymicrobial synergy and dysbiosis model, in which several low-abundance microbes synergistically promote a remodeling of the oral microbiome in order to attack the host's defenses (3). Among the identified species in this process, *Treponema denticola*, a Gram-negative oral spirochete, has been implicated in the advanced or aggressive forms of the disease and in periodontal disease recurrence (1, 2). Our group recently reported that *T. denticola* mediates cytoskeletal dysfunction and upregulation of tissue destructive genes in human-derived periodontal ligament (hPDL) cells via RASA4 (2) and TRL2/MyD88 (1) pathways, respectively.

Periodontal disease is currently managed by mechanical removal of the bacterial plaque (scaling and root planning). Given the chronic and episodic nature of the disease and bacterial nature of dental plaque biofilms, the use of topical and systemic antibiotics have been increasingly used in the field to treat the infection (10). However, microbes are known to undergo internalization into host cells and thereby avoid the actions of antimicrobials, causing persistent and recurrent infections (11,12).

The advent of multidrug-resistant microbes and the scarcity of new antibiotics, which are growing concerns for overall human health (3,13), further challenge current protocols for periodontal disease treatment. The current crisis of antimicrobial resistance has been attributed to the overuse/misuse of known antibiotics, prompting the rise of multidrug resistant organisms, especially bacteria. Furthermore, the unavailability of new antimicrobials has been attributed to the declining private investments in the field, which culminates in a lack of innovation and termination of current antibiotic research and development (R&D) programs by pharmaceutical companies (13–15). Therefore, comprehensive efforts are necessary to address these global issues impacting overall health, including oral health by studying and leveraging emergent microorganisms/probiotics and their antimicrobial peptides (13).

Antimicrobial peptides (AMPs) are emerging antimicrobial agents produced by several bacterial phyla, plants, animals, and even humans (3,13). These peptides are known for their usual broad spectrum of action, i.e., active against both gram-positive and negative bacteria, and even certain fungi and protozoan species (3,13). Although most AMPs demonstrate antimicrobial activity due to cellular membrane damage, some can also target certain proteins, and the DNA and RNA of their target species, making them highly suitable as replacements for classical antibiotics (3,13). In light of this, our group previously explored an AMP, nisin, and demonstrated that this bacteriocin produced by Lactococci species and currently used in food preservation (16), is an effective antimicrobial. It inhibits key periodontopathic bacteria, including *T. denticola* (17,18), and disrupts oral biofilms in in vitro (17–19) and in vivo (20) periodontal disease models, as well as in periimplantitis models (21). Additionally, nisin also shows anticancer activity against oral squamous cell carcinoma in vitro and in vivo (9,22,23), without cytotoxic effects to normal oral cells (18). Given the potential use of nisin in the management of periodontal disease and in other biomedical uses (24), nano-sized drug delivery systems can be used to optimize and/or enhance nisin capabilities.

Nano-sized drug delivery systems (nano-DDS) are devices or formulations sized in the nanometric range that modify and improve a therapeutic agent's efficacy and safety by controlling the rate, time, and/or selectivity of drug release in the body (25,26). Additionally, nano-DDS can be designed to modify the pharmacokinetics and biodistribution of therapeutic agents, to protect agents from degradative enzymes, and to act as drug reservoirs in the targeted tissue (16). Among these systems, Solid Lipid Nanoparticle (SLN) constitute one of the promising candidates. SLN are nanoparticles made of biocompatible solid lipids at room temperature; they exhibit high stability (>2 years); and scalability (27–32). Recently, our group showed that nisin-loaded SLN (SLN-Nisin) can significantly inhibit the growth of planktonic *Treponema denticola*, disrupt oral biofilms, and decrease oral squamous cell carcinoma cell (OSCC) viability compared to free nisin (30).

Therefore, the objective of this work was to test whether SLN can effectively deliver nisin and treat *Treponema denticola*-infected human-derived hPDL cells.

Chapter 3.3: RESULTS

We first analyzed *T. denticola* internalization kinetics in hPDL cells (Figure 3.3.1). An increasing amount of *T. denticola* internalization was observed over time in the hPDL cells (Figure 3.3.1A). A significant increase in the percentage of *T. denticola*-positive cells over time compared to the control was also noted (Figure 3.3.1B), although no statistical difference was found among the different time points. In addition, *T. denticola* internalization intensity significantly increased over time with significant differences after 12h and 24h compared to control (Figure 3.3.1C). In order to evaluate whether the *T. denticola* bacteria were indeed inside the hPDL cells or only on the surface, Z-stacks for 12h and 24h (Movie S3.7.1 and S3.7.2) were undertaken. We 3D reconstructed the Z-stacks (Movie S3 and S4) and analyzed the fluorescence intensity slice by slice of the hPDL cell nuclei and *T. denticola* genetic material (Figure S3.7.1). Our data indicate that *T. denticola* was indeed intracellular, and not merely bound to the outer surface of the hPDL cell membrane.

Next, we investigated the pathway by which *T. denticola* undergoes internalization and therefore tested bacterial colocalization with Rab5, an early endosome marker [37,40] (Figure 3.3.2). We found that *T. denticola* colocalized with Rab5 on an average of 662.5±211.3 and 758.0±157.6 colocalization sites after 4h and 5h of infection, respectively. The bacterium, then, significantly increased to 1548.0±165.0 colocalization sites after 8h of infection (Figure 3.3.2B) and significantly decreased to 326.0±142.8 colocalization sites after 9h of infection. It is noteworthy that *T. denticola* can be detected inside or very close to the hPDL cells nucleus by 6h

After evaluating the involvement of the early endocytosis pathway, we, evaluated whether *T. denticola* colocalizes with Rab7, a late endocytosis marker (37,41, 42) at 9h and 10h of infection (Figure 3.3.3). We observed 1290±498.5 colocalization sites at 9h between the bacterium and the late endosome

marker. The number of colocalization sites further increased to 5453±300.5 at 10h of infection, indicating further progression of the bacterium through the endocytic pathway.

In order to evaluate the possible changes mediated by *T. denticola* in hPDL cells within the initial 24h of infection, we analyzed the hPDL cell gene expression following bacterial infection (Figure 3.3.4). *T. denticola* significantly altered the expression of 560 genes within the first 5h of infection and 2103 genes at 24h (Figure 3.3.4A). Principal component analysis (PCA; Figure 3.3.4B), indicated a complete cluster separation between the two time points. Additionally, the 24h cluster showed a higher variance (larger ellipse) compared to the 5h cluster. Using a volcano plot analysis, we identified the 52 most significantly altered genes, such that 36 were down-regulated and 16 were up-regulated.

These data indicate that once *T. denticola* is internalized, the bacterium significantly alters hPDL gene expression within hours of infection. Given this time-dependent finding, we used SLN-Nisin to eliminate intracellular *T. denticola* before it exerts its effects on hPDL gene expression. SLN-Nisin has been characterized before [30]. Briefly, SLN-Nisin has a diameter of 205±7nm and a polydispersity index (PDI) of 0.329±0.04, whereas the unloaded Nano-DDS has a diameter of 105 ± 1 nm and a PDI of 0.052 ± 0.013 [30]. Therefore, we next evaluated the timing of SLN-Nisin's internalization (Figures 3.3.5A and 5B). 89.47%±2.76% of hPDL cells were positive for SLN-Nisin at 1h (Figure 3.3.5A). This number slightly increased to 99.60%±0.42% after 8h of internalization. Regarding SLN-Nisin internalization staining intensity (Figure 5B), our results showed a bell-shaped curve with the intensity starting at 656,596±74,759 A.U. at 1h, then increasing to 1,317,222±118,192 A.U. at 4h, and remaining constant at 6h before decreasing back to 1,117,768±71,606 A.U. at 8h. Since SLN-Nisin internalization intensity peaked at 4h (no significant difference compared to 6h), we established 4h as the standard SLN-Nisin internalization time for treatment of hPDL cells henceforth.

After determining this internalization time, we, next evaluated SLN-Nisin's progression through the endocytic process (Figure 3.3.5C and 5D) by using specific endocytosis pathway inhibitors. For cells positive for SLN-Nisin (Figure 3.3.5C), no statistical differences were observed among the inhibitors. In contrast, SLN-Nisin internalization intensity for clathrin-mediated endocytosis and phagocytosis were significantly decreased by half (to 188,507±33,012 or 47.42% relative to no inhibitor control, respectively) and by a fourth (to 263,681±16,096 or 26.45% relative to no inhibitor control, respectively), compared to control (358,529±31,879 A.U.). This specific result indicates that SLN-Nisin is significantly (73.87%) internalized by clathrin-mediated endocytosis and phagocytosis in hPDL cells.

We next tested whether nisin and SLN-Nisin are able to inhibit *T. denticola* internalization into hPDL cells and the results can be seen in Figure 3.3.6. No significant difference was observed for *T denticola*-positive cells for both treatments (Figure 3.3.6A). However, both nisin and SLN-Nisin were able to significantly reduce *T. denticola* internalization intensity from 66,018±10,323 A.U. to 38,538±1,589 A.U. and 44,095±11,590 A.U., respectively. This meant that nisin and SLN-Nisin reduced *T. denticola* internalization intensity by about 40% and 33%, respectively, and significant differences were observed between both treatments.

Given the potential for SLN-Nisin to treat intracellular *T. denticola* infection and since both (the bacterium and SLN-Nisin) are internalized via endocytic pathways, we hypothesized that SLN-Nisin might colocalize with *T. denticola* inside hPDL cells in late endosomes. To do this, we used Rab7 as a late endosome marker [37] to test our hypothesis and the results can be seen in Figure 3.3.7. After 10h of infection (timepoint where *T. denticola* internalization peaks at the late endosome), we detected the combined colocalization of *T. denticola*, SLN-Nisin and Rab7 (Figure 3.3.7A). On average, we detected 6,822 ± 77 colocalization sites related to Rab7 and *T. denticola*; 5,744 ± 117 colocalization sites related to Rab7 and SLN-Nisin; 6,173 ±185 colocalization sites related to *T. denticola* and SLN-Nisin; and, finally, 4,661±677

colocalization sites related to the triple colocalization (Figure 3.3.7B). Normalizing these values to the Rab7-*T. denticola* number of colocalization sites, we observed that 84.2±1.7% of the colocalizations were related to Rab7 and SLN-Nisin; 90.49±2.7% were related to *T. denticola* and SLN-Nisin; and 68.3±9.9% were related to the triple colocalization. No significant difference between the double and the triple colocalizations was found after the normalization.

After establishing that *T. denticola* and SLN-Nisin colocalize to the same vesicles (i.e., late endosomes), we hypothesized that SLN-Nisin might reduce the *T. denticola* content inside of hPDL cells. We previously observed that *T. denticola* DNA material remains inside of hPDL cells for at least 6 days (data not shown). Therefore, we tested whether nisin and SLN-Nisin can reduce *T. denticola* content after 48h of infection (Figure 3.3.8). Our results showed that SLN-Nisin containing 13µg/mL and 26µg/mL of nisin significantly reduced the number *T. denticola*-positive hPDL cells compared to control down to 14.2 ± 3.8% and 2.9 ± 0.48%. Compared to the same concentration of free nisin (13µg/mL), we observed that SLN-Nisin significantly increased nisin efficacy in removing *T. denticola* DNA by 6.5-fold. No significant difference was found for 13µg/mL of free nisin and SLN-Nisin containing 6.5µg/mL of nisin. Similarly, the fluorescence intensity showed a significant decrease in *T. denticola* content to 8.1 ± 1.6 % and 1.5 ± 0.3% for SLN-Nisin containing 13µg/mL and 26µg/mL of nisin. Likewise, SLN-Nisin increased nisin efficacy in decreasing *T. denticola* content by 12-fold, compared to the free nisin counterpart control (13µg/mL).

Finally, we then tested whether SLN-Nisin treatment was able to revert 4 of the top altered gene expressions (i.e., IL1R1, GRIN2A, STC1 and IL-6) by *T. denticola* and the results can be seen on Figure 3.3.9. *T. denticola* infection significantly increases GRIN2A levels, while significantly reduces IL1R1, STC1 and IL-6 gene expression levels compared to control, thus validating our RNA-seq data. Both Nisin and SLN-Nisin were, then, able to significantly increase IL1R1 and decrease GRIN2A levels compared

to infection, turning them similar to control levels (Figure S2). Remarkably, only SLN-Nisin was able to significantly increase levels of STC1 and IL-6 compared to infection, turning them similar to control levels (Figure S2).

Chapter 3.4: DISCUSSION

Periodontal disease is a complex bacterially-mediated inflammatory disease. A consortia of bacteria that include *Treponema denticola* are thought to act in synergy to subvert and overcome the host immune response (3). To date, periodontal disease is treated with scaling, root planning, surgery, and local or systemic antibiotics (43.) However, microbes are known to undergo internalization into host cells, thereby avoiding eradication by antimicrobial therapy and causing persistent and recurrent infections (11,12). For the particular case of *T. denticola*, Inagaki et al. (44) and Shin & Choi (45) reported that the bacterium can invade human gingival epithelial cells. Furthermore, Miao et al. (46) reported the internalization of *T. denticola* by hPDL cells; the latter demonstrated after 24h of infection by colocalization with LAMP1, a lysosomal marker (37). In both cases, the bacterium was able to survive antibiotic treatments while internalized in the host cell, and our results validate that the bacteria is detected intracellularly in hPDL cells.

Additionally, Inagaki et al. (44) demonstrated that a *T. denticola* dentilisin-deficient mutant had significantly lower internalization potential with respect to gingival epithelial cells, indicating that dentilisin may be involved in the internalization process. The authors further investigated the mechanism of *T. denticola* internalization and demonstrated that the bacterium was internalized via a cholesterol-dependent mechanism, such as caveolin-mediated endocytosis. Although the involvement of dentilisin in *T. denticola* internalization by hPDL cells and the specific pathway involved still need to be further explored, our study also indicates that a similar endocytic pathway is implicated since early and late endosomes are involved in the transportation of the bacterium, rather than a phagosome for instance.

Furthermore, reports indicate that *T. denticola* numbers slowly decay over time inside of host cells. Miao et al. (46) reported that the bacterium survived for 7h but did not survive up to 24 h inside of hPDL

cells. In addition, Shin & Choi (45) showed that although a culture-based antibiotics protection assay could not detect viable intracellular *T. denticola* 12 h after infection, a substantial number of bacteria were observed by confocal microscopy and weak expression of bacterial 16S ribosomal RNA was detected 48 h after infection. In addition, flow cytometric analysis of epithelial cells infected with labeled *T. denticola* showed no loss of fluorescence over 48 h. In addition to *T. denticola* survival and persistence inside of host cells, our observations further indicate that *T. denticola* significantly alters gene expressions (both up- and down-regulation) in hPDL cells by 24 h. This shows how quickly the bacterium is able to subvert and manipulate hPDL cell gene expression. Given these time-sensitive effects, we considered approaches to eliminating internalization by and intracellular effects of *T. denticola*. In this context, we tested SLN-Nisin for its ability to abrogate *T. denticola* internalization and entry into the endocytic pathway as a means of blocking the *T. denticola*-mediated alterations in gene expression in hPDL cells.

SLN have been shown to be safe in vitro (29) and in vivo (32). Recently, we demonstrated that SLN-Nisin can improve nisin's efficacy in inhibiting the growth of planktonic *T. denticola*, as well as in disrupting saliva-derived oral biofilms, and in mitigating cancer processes in oral squamous cell carcinoma (OSCC) cells up to 10-fold (30). Although most common antibiotics cannot effectively access internalized bacteria (11), we demonstrated in this work that SLN-Nisin can significantly reduce the internalized *T. denticola* intensity below 10% compared to control. Moreover, SLN-Nisin was able to significantly reverse 4 of the top altered genes by *T. denticola* infection back to control levels, which highlights a potential clinical relevance as periodontal disease treatment.

Previous reports indicated that the same SLN formulation complexed with DNA was internalized via clathrin-mediated endocytosis in human embryonic kidney (HEK) cells (28) and caveolin-mediated endocytosis in prostate cancer (PC3) cells [36], indicating that the nanoparticle's internalization pathway is cell line/type-dependent. For hPDL cells, SLN-Nisin is primarily internalized by both

phagocytosis and clathrin-mediated endocytosis. Phagocytosis is commonly classified as the cellular uptake process for particles larger than 500 nm (37,47–49), with its phagosome diameter ranging from 0.5 to 10μm (50). Endocytosis, on the other hand, is classified as the uptake mechanism for particles smaller than 500nm (37,47–49), with clathrin-mediated endocytosis vesicles having a diameter of about 100nm (50). In the particular case of hPDL cells, both phagocytosis and clathrin-mediated endocytosis processes uptake nanoparticles with an average size of 205±7nm of diameter. Phagocytosis is likely used to clear several nanoparticles at once, whereas clathrin-coated vesicles internalize nanoparticles one by one or in more limited numbers.

We were able to colocalize both SLN-Nisin and *T. denticola* inside of PDL cells at the late-endosome stage. Forty-eight hours later, SLN-Nisin significantly reduced the number of cells positive for *T. denticola* and the *T. denticola* intensity, compared to free nisin. Further, we were able to significantly revert 2 and 4 of the top *T. denticola*'s altered gene expression by using Nisin and SLN-Nisin, further demonstrating the potential treatment capabilities of both formulations. To the best of our knowledge, this is the first demonstration of the ability of a nisin-loaded nanoparticle to mitigate microbial internalization and infection.

Chapter 3.5: MATERIALS AND METHODS

Ethics statement

Approval to conduct human subjects' research, including protocols for the collection and use of human teeth and PDL tissue was obtained from the University of California San Francisco Institutional Review Board (#16–20204; reference #227030). Consent was not obtained due to anonymity of the samples.

Human-derived periodontal ligament (hPDL) cell culture

The isolation and culture of human-derived periodontal ligament (hPDL) cells was conducted as previously described (1,2). Briefly, periodontal ligament tissues were isolated from the middle third of extracted healthy human teeth and maintained for cell outgrowth in Minimum Eagle Medium-α (MEM-α) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin/streptomycin, and 1% amphotericin B (Gibco, USA) in a Forma II incubator (Thermo-Fisher Scientific, USA) at 37℃ with a humid atmosphere containing 5% CO2. Cell outgrowths were passaged before reaching confluency. Only cells passaged three to seven were used for experimentation and cells were validated via morphological assessment.

Treponema denticola cell culture

Treponema denticola (ATCC 35405) was grown at 37℃ in Oral Treponeme Enrichment Broth (OTEB; Anaerobe Systems, USA) inside an anaerobic chamber filled with an anaerobic air gas mixture (replaces O2 gas volume for N2). Cells were passaged every 5-7 days.

hPDL cells challenged with *Treponema denticola*

After seeding, the hPDL cells were challenged with wildtype *T. denticola*, as previously described (1,2,33). Briefly, the *T. denticola*-treated cells were centrifuged at 4000 RPM for 15 minutes and the supernatant was removed. The pelleted bacterial cells were then resuspended in serum- and antibiotic-

free MEM-α (without phenol red) at an optical density (OD) of 0.1 at 600 nm as assessed with a Spectramax M2 microplate spectrophotometer (Molecular Devices, USA). It has been previously established that an OD of 0.1 at 600nm is equivalent to 2.4x108 CFU/ml of *T. denticola* (34). Next, hPDL cells were challenged with the bacteria at 50 multiplicities of infection (MOI) in serum- and antibiotic-free media (no phenol red MEM-α; Gibco, USA) and incubated for 2h at 37℃ and 5% CO2. Previous reports have indicated that a 50 multiplicity of infection (MOI) rendered a strong cellular response in hPDL cells without inducing any cytotoxic effects in the cells (2). Cells were then washed with PBS three times to remove any unbound bacterial cells.

RNA-Seq

hPDL cells (9.5x105 cells/well) were challenged with *T. denticola* as described above for either 5h or 24h. Then, RNA was extracted from the samples using a RNeasy Mini Kit (Qiagen, Germany) and following the manufacturer's instructions. The quality of the extracted RNA was assessed using a Nanodrop UV-Vis Spectrophotometer (Thermo-Fisher Scientific, USA) and by calculating the percentage of RNA fragments with size > 200 bp using a 2100 Bioanalyzer (Agilent, USA). The RNA was used for first and second strand synthesis, polyA tail bead capture, and sequencing adapter ligation using a TruSeq RNA Library Prep Kit v2 (Illumina, USA). Libraries were sent to Novogene Genomic Services (USA) for paired-end sequencing on a HiSeq 4000 instrument (150 bp paired-end reads; Illumina, USA) and analysis.

SLN and SLN-Nisin production

SLN production has been described before (28,29,31,32,36). Briefly, stearic acid (7 mM, Sigma-Aldrich, USA), DOTAP (2.5 mM, Avanti Polar Lipids, USA) and Pluronic F68 (1 mM, Sigma-Aldrich, USA) were added to a test tube, heated to 75°C and extruded through a 75°C heated polycarbonate membrane (pore size: 0.1 μm) apparatus. The resultant volume was put in an ice bath until cooled, then stored at 4°C until use.

The production of nisin-loaded SLN (SLN-Nisin) has also been described before (30) and it is a modification of the SLN production method described earlier to incorporate nisin into the lipid matrix. Briefly, a 5 mg/mL solution of nisin (Handary, Belgium) in Mili-Q water was produced. Then, the solution was agitated on a rotor for 4h and filtered using a 0.22µm filter (Millipore, USA). During the nanoparticle production, 3.25mg/mL of the filtered nisin solution was added to the lipid mixture before extrusion. Labeled SLN and SLN-Nisin were produced by adding 0.4 mol% of rhodamine-PE (Avanti Polar Lipids, USA) to the lipid mixture before extrusion.

SLN-Nisin internalization and kinetic assay

The SLN-Nisin internalization kinetics assay was executed as described earlier (28). Briefly, 1x105 cells/well of hPDL cells were seeded onto 6-well plates and allowed to adhere overnight. The next day, the cells were washed in PBS and 1mL of antibiotic- and serum-free MEM-α (no phenol red) medium containing 4µL of labelled SLN-Nisin was added to the cells for 0.5, 1, 2, 4, 6 and 8h. Then, the hPDL cells were washed in PBS, trypsinized and analyzed using a FACScalibur flow cytometer (BD, USA).

SLN-Nisin endocytosis pathway inhibition assay

The SLN-Nisin endocytosis pathway inhibition assay was executed as described earlier (28,37). Briefly, 1x105 cells/well of hPDL cells were seeded into 6-well plates and allowed to adhere overnight. The next day, the cells were washed in PBS and incubated for 30min with one of the endocytosis inhibitors (Table 1) in MEM-α (no phenol red). Then, 4µL of labelled SLN-Nisin was added to the top of the inhibitor-containing media and then cells were incubated for 4h more. Finally, the hPDL cells were washed in PBS, trypsinized and analyzed using a FACScalibur flow cytometer (BD, USA).

Table 1 – Endocytosis inhibitors used

Compound	Endocytosis pathway inhibited	Concentration
Cytochalasin D	Phagocytosis	5µg/mL
Chlorpromazine	Clathrin-mediated	1µg/mL
Methyl-B-Cyclodextrin	Caveolin-mediated	12.5µg/mL
Wortmannin	Macropinocytosis	13µg/mL

Treponema denticola internalization kinetic assay

hPDL cells (3x104 cells/well) were seeded onto 4-well chamber slides (Invitrogen, USA) and allowed to adhere overnight. Syto 9 staining has been used previously in bacterial culture assays with *T. denticola* and the stain did not interfere with the functioning or viability of the pathogen (2,38). HPDL cells were challenged *T. denticola* (as described earlier) and incubated for 0, 2, 6, 12 and 24h after challenge. Post-challenge, cells were incubated with gentamicin (1:1000) as previously described (46). Chamber slides were washed with PBS, and fixed with 4% PFA. Slides were incubated with 1% Trition X-100 and probed with anti-MSP rabbit antibodies 1:1000 concentration (2 hours) followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:4000; Abcam, USA) and Hoechst 33342 (1:3000; Millipore-Sigma, USA) nuclear for 4 hours. Finally, the hPDL cells were imaged using a Leica SP8 X Confocal Laser Scanning Microscope. Images were processed using FIJI/ImageJ imaging analysis software. Anti-Msp antibody was obtained from Fenno Lan and has been confirmed to be specific to *Td* derived Msp protein. (51)

Inhibition of *Treponema denticola* internalization by SLN-Nisin

hPDL cells (1x105 cells/well) were seeded onto 6-well plates and allowed to adhere overnight. The next day, *T. denticola* cells were stained for 20 mins with a nuclear dye Syto 9 (1:1000), vortexed and washed with MEM-α (no phenol red) media twice to remove the excess dye. Next, the hPDL cells were

challenged with the Syto-9-stained bacteria, as described earlier. Then, the infected hPDL cells were treated with 13µg of nisin, in the form of free nisin or SLN-Nisin for 4h. Finally, the hPDL cells were washed in PBS, trypsinized and analyzed using a FACScalibur flow cytometer (BD, USA).

Treponema denticola and SLN-Nisin colocalization with endocytosis markers

hPDL cells (3x104 cells/well) were seeded onto 4-well chamber slides (Invitrogen, USA) and allowed to adhere overnight. The next day, the cells were transduced overnight with lentiviral vectors for either Rab 5-RFP (CellLight Early endosomes-RFP BacMam 2.0; ThermoFisher, USA) or for Rab7-GFP (CellLight Late endosomes-GFP BacMam 2.0; ThermoFisher, USA). Then, T. denticola cells were stained for 20 mins with the nuclear dye Syto 9 (1:1000), vortexed and washed with MEM-α (no phenol red) media twice to remove the excess dye. Next, hPDL cells were challenged with the Syto-9-stained bacteria, as described earlier. 6h after *T. denticola* infection, hPDL cells were treated with 4µL of labelled SLN-Nisin. Finally, the hPDL cells were stained with Hoescht 33342 (1:2000; Millipore-Sigma, USA) nuclear stain and imaged using a Leica SP8 X Confocal Laser Scanning Microscope.

The resulting images were analyzed via object-based colocalization within the hPDL cells using ImageJ/Fiji with the JaCoP plugin. For pictures capturing both hPDL cell nuclei and *T. denticola* genetic material, hPDL nuclei were manually removed from the images before quantification of fluorescence intensity. For triple colocalization (SLN-Nisin, Treponema denticola and Rab7), two out of the three channels were selected randomly for the initial colocalization, as described earlier, except for selecting the plugin to show the center map. The center map is an image generated by the plugin with only the colocalized objects in the picture (and removing the remainder). After the initial analysis, the center map image was used for the second and final colocalization analysis with the third channel to obtain the triple object colocalization count.

Treponema denticola DNA clearance

hPDL cells (3x104 cells/well) were seeded onto 4-well chamber slides (Invitrogen, USA) and allowed to adhere overnight. The next day, the hPDL cells were challenged with *T. denticola* for 2h, as described earlier. Then, hPDL cells were treated with either nisin (13µg/mL) or SLN-Nisin (concentrations equivalent to 6.5, 13 or 26µg/mL of nisin) and incubated for an additional 46h. Finally, *T. denticola* and hPDL cellular DNA were stained using Hoescht 33342 (1:2000; Millipore-Sigma, USA) and imaged using a Leica SP8 X Confocal Laser Scanning Microscope.

hPDL Gene Expression

hPDL cells (5x105 cells/well) were seeded onto 6-well plates (Corning, USA) and allowed to adhere overnight. The next day, the hPDL cells were challenged with *T. denticola* for 2h, as described earlier. Then, hPDL cells were treated with either nisin (13µg/mL) or SLN-Nisin (concentrations equivalent to 6.5, 13 or 26µg/mL of nisin) and incubated for an additional 22h. On then next day, hPDL cells were harvested for RNA extraction using RNeasy Micro Kit (Qiagen, USA) accordingly to the kit's manual. Next, cDNA library was synthesized by using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo-Fisher, USA) according to the kit's manual using a Quantstudio 3 (Thermo-Fisher, USA). The top 4 gene expression levels altered by *T. denticola* by fold change and p-value (i.e., IL1R1, GRIN2A, STC1 and IL-6 – indicated by the RNA-Seq) were, then, selected and tested via qPCR using a Quantstudio 3 (Thermo-Fisher, USA).

Statistical analysis

All data was analyzed using GraphPad Prism software (GraphPad, USA) and the results were evaluated by one-way ANOVA and displayed as mean□S.D. For the RNA-Seq data, PCA analysis was performed via the web tool ClustVis (39), while the volcano plot analysis was performed using RStudio Software (RStudio, Austria).

Chapter 3.6: FIGURES

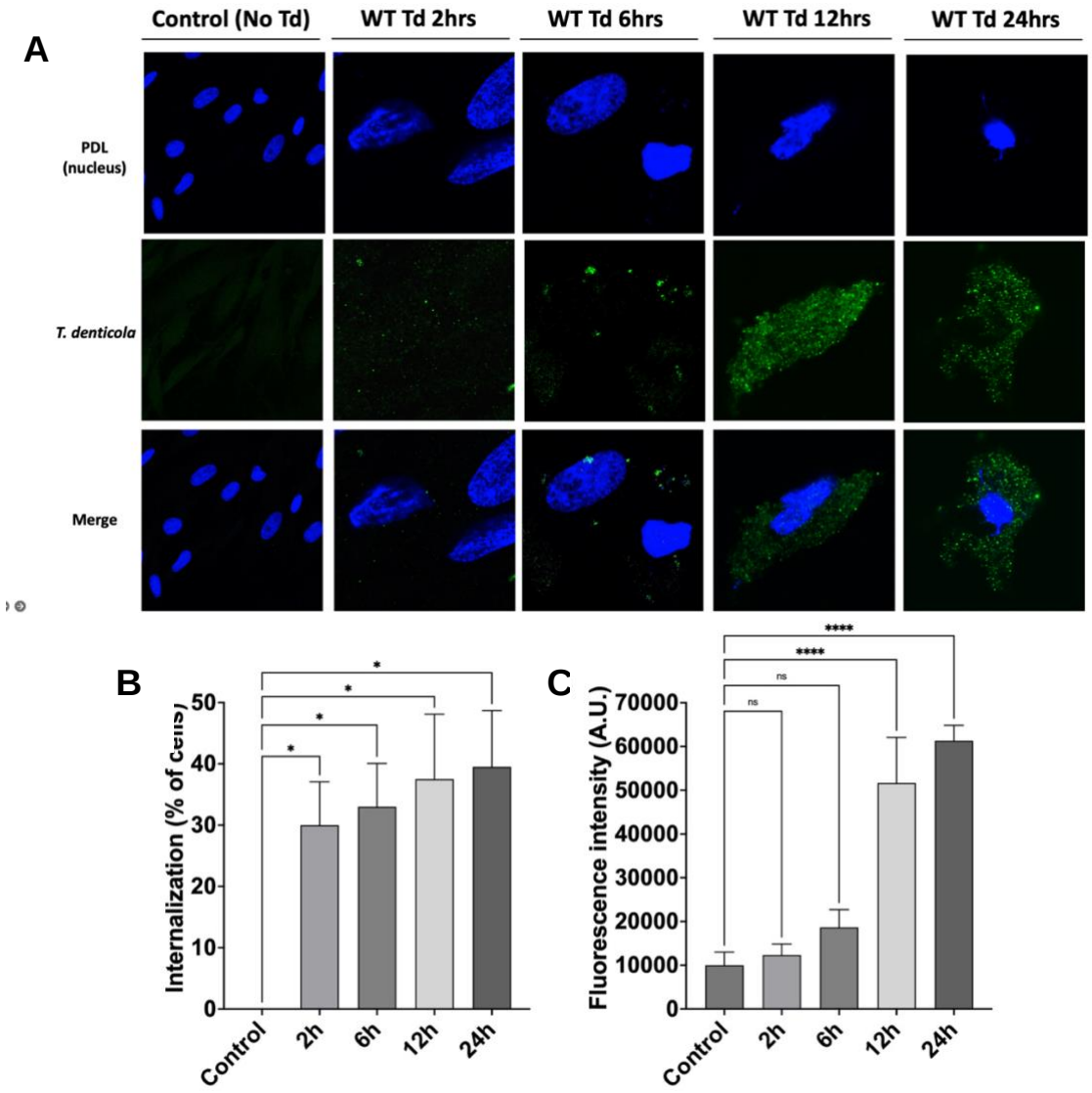


Figure 3.3.1. *Treponema denticola* is significantly internalized by hPDL cells after 12h and 24h. (A) Confocal microscopy of hPDL nuclei (Top row; blue) and *T. denticola* (Middle row; Green) and the merged image (bottom row). Columns indicate the time points from 0h (Control; left) to 24h (right). White arrows highlighting *T. denticola* detected inside or very close to hPDL cell nucleus. (B) Semi-quantification of the images was expressed as percentage of *T. denticola*-positive cells showing internalization over time. (C) Semi-quantification of the images using fluorescence intensity of the *T. denticola* channel over time. * means $p \leq 0.05$ between marked samples **** means $p \leq 0.0001$ between marked samples.

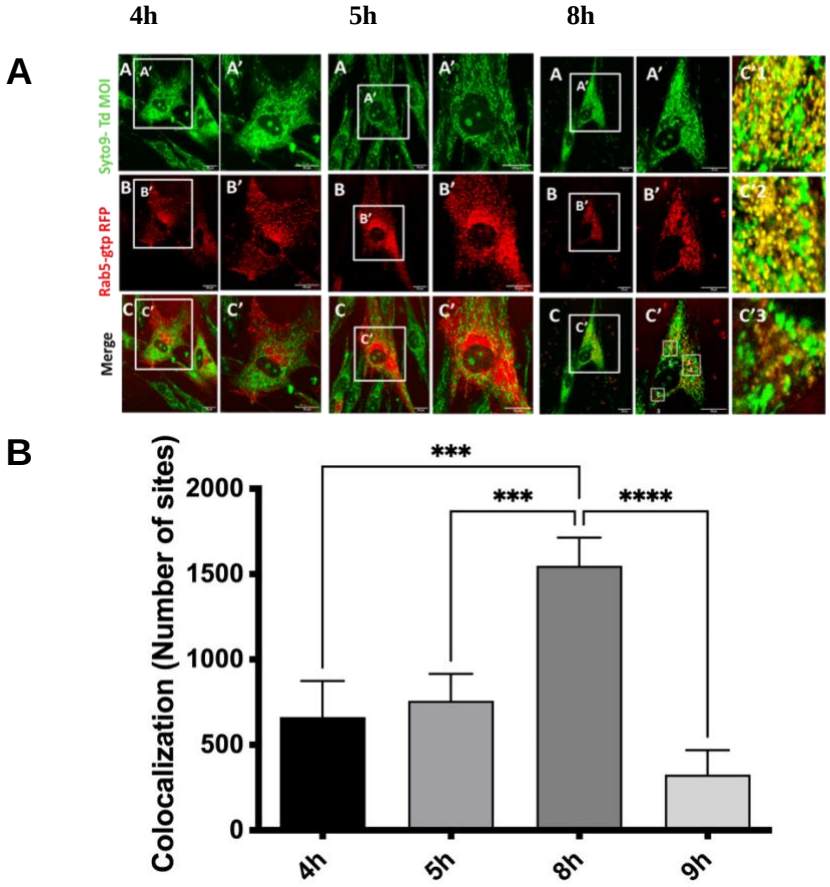


Figure 3.3.2. *Treponema denticola* colocalizes with the early endocytosis marker Rab5 and this colocalization significantly peaks at 8h post challenge. (A) Confocal microscopy of *T. denticola* (Top row; Syto9; green) and hPDL Rab5 (Middle row; Red) and the merged image (bottom row). Columns indicate the time points from 0h (Control; left) to 24h (right). (B) Number of colocalizations in the images. *** means $p \leq 0.001$ between marked samples; **** means $p \leq 0.0001$ between marked samples.

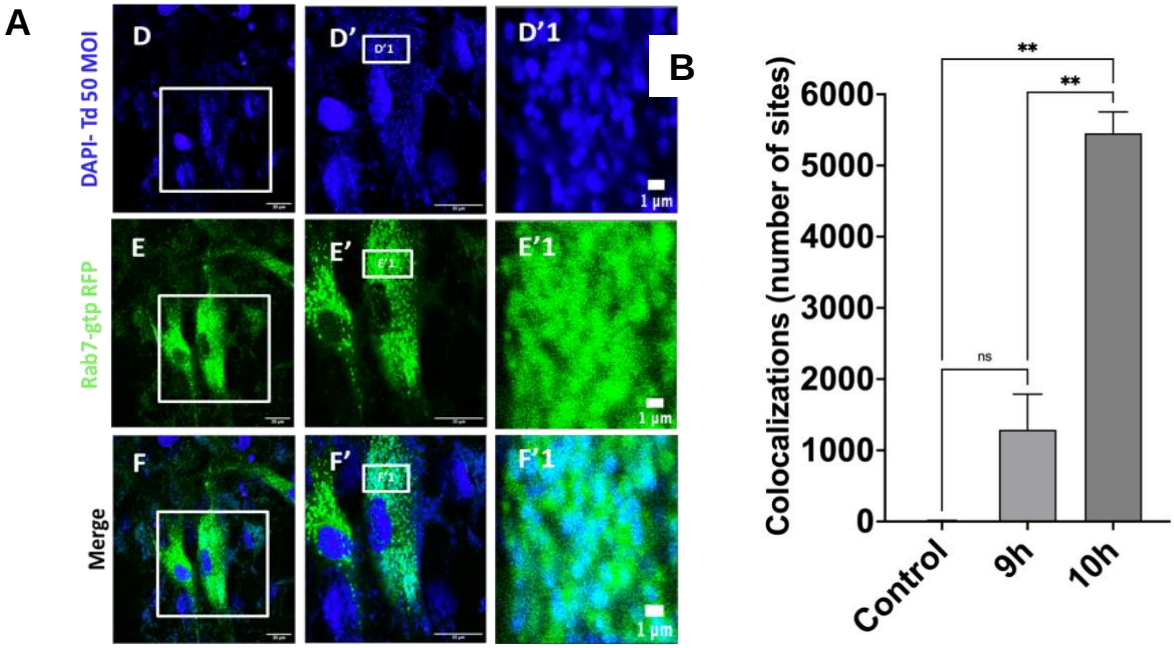


Figure 3.3.3. *Treponema denticola* colocalizes with late endocytosis marker Rab7 and significantly peaks at 10h post challenge. (A) confocal microscopy of *T. denticola* (Top row; DAPI; Blue) and hPDL Rab7 (Middle row; Green) and the merged image (bottom row). Columns indicate the time points from 0h (Control; left) to 24h (right). (B) Number of colocalizations in the images. *** means $p \leq 0.001$ between marked samples.

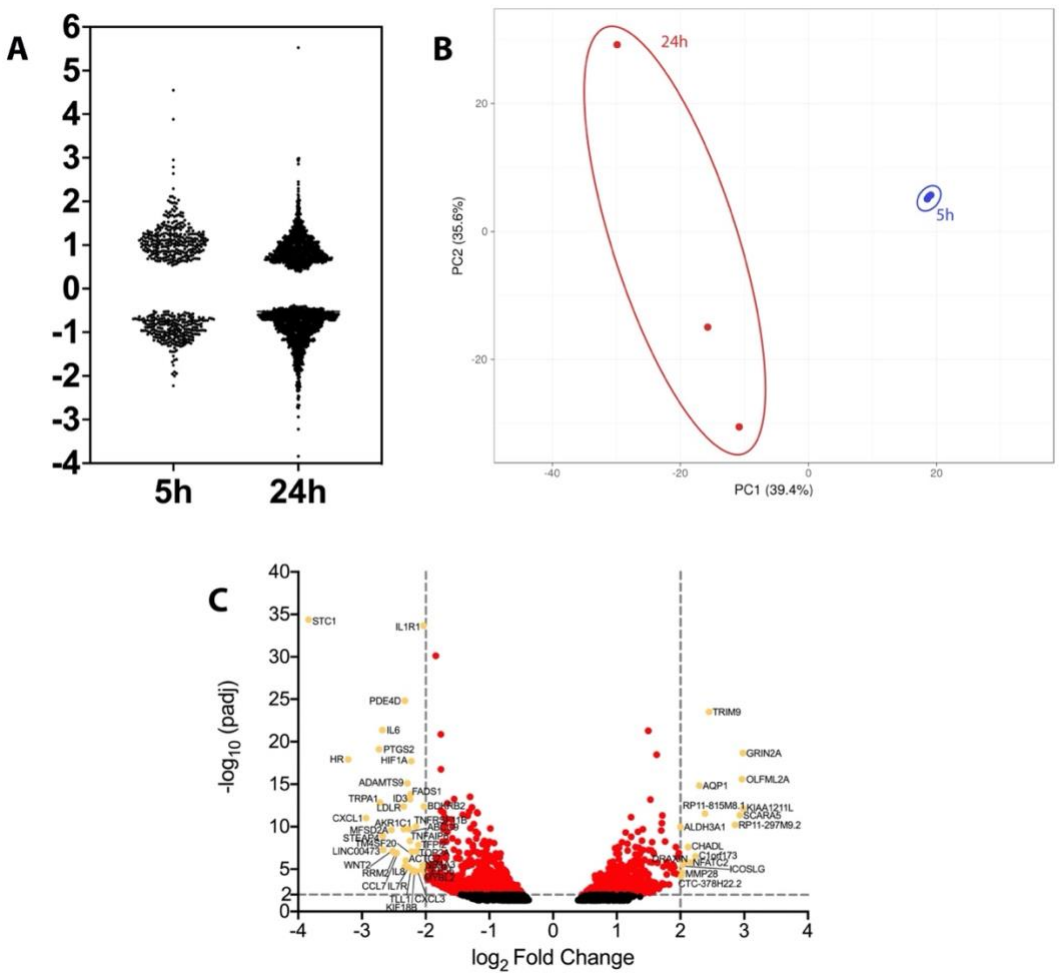


Figure 3.3.4. *Treponema denticola* significantly up- and down-regulates over 1,500 hPDL genes from 5h to 24h. (A) Log_2 fold change of the genes altered by *T. denticola* after 5h and 24h of infection, compared to control (no infection). (B) Principal component analysis (PCA) of the genes altered by *T. denticola* after 5h and 24h of infection. (C) Volcano plot of the gene expression changes caused by *T. denticola* invasion of hPDL cells. Red signifies genes with adjusted p-values > 2; Blue signifies genes with Log2 fold change > 1; Gold signifies both cases. Highlighted are the most significant altered genes expression by *T. denticola* infection.

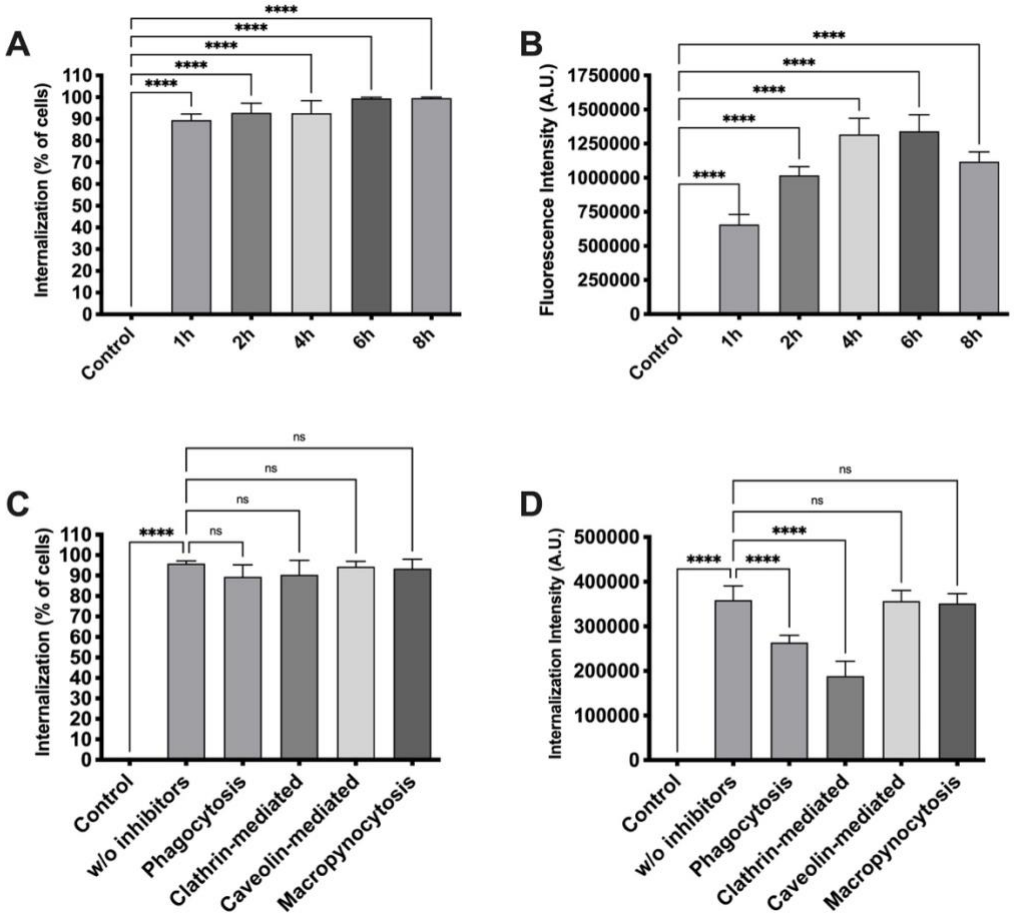


Figure 3.3.5. SLN-Nisin undergoes internalization into periodontal ligament cells via endocytic mechanisms. (A) Quantification of positive hPDL cells for SLN-Nisin internalization over time. (B) Fluorescence intensity of internalized SLN-Nisin over time. (C) Quantification of positive hPDL cells for SLN-Nisin internalization with or without inhibitors for phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and macropinocytosis. (D) Fluorescence intensity of internalized SLN-Nisin with or without inhibitors for phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and macropinocytosis. **** means $p \leq 0.0001$ between marked samples.

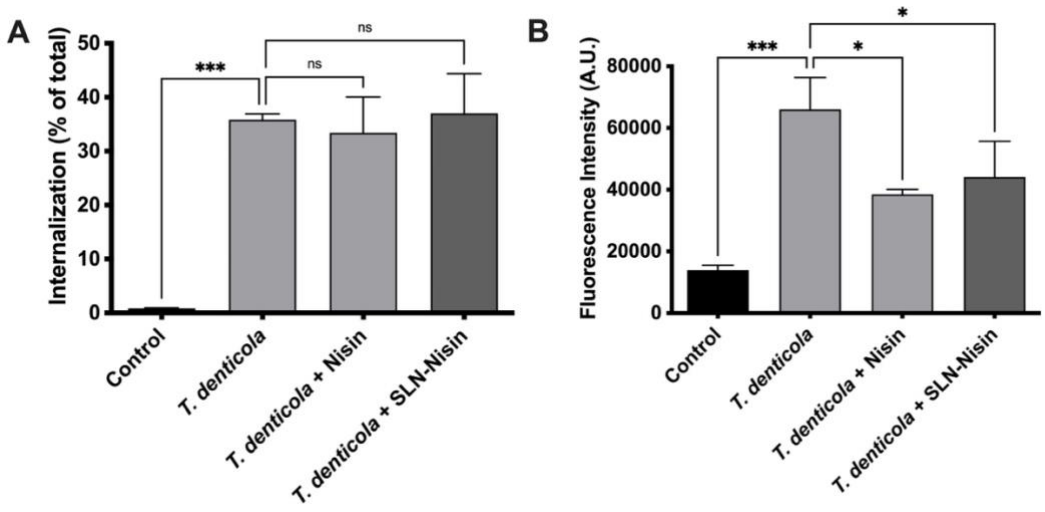


Figure 3.3.6 Nisin and SLN-Nisin reduces *Treponema denticola* internalization into hPDL cells. (A) Quantification of *T. denticola*-positive hPDL cells treated with either nisin or SLN-Nisin. (B) Fluorescence intensity of *T. denticola* in hPDL cells treated with either nisin or SLN-Nisin. * means $p \leq 0.05$ between marked samples. *** means $p \leq 0.001$ between marked samples.

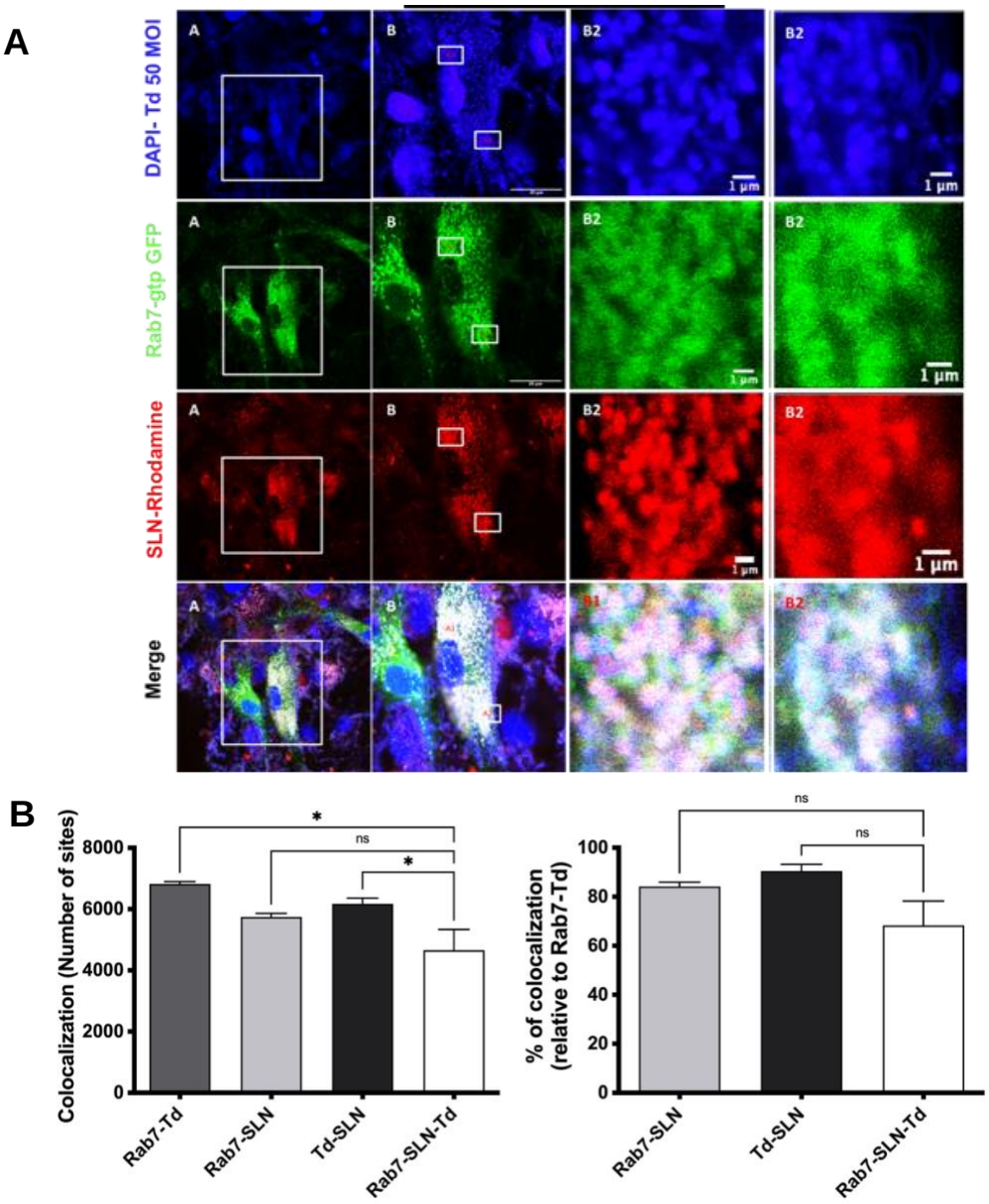


Figure 3.3.7 SLN-Nisin colocalizes with Rab7 and *T. denticola* 10h after challenge. (A) Confocal microscopy of *T. denticola* (Top row; DAPI; Blue), hPDL Rab7 (Middle-upper row; Green), SLN-Nisin (Middle-lower row; Red) and the merged image (bottom row). (B) Number of colocalizations in the images. (C) Percentage of colocalizations relative to Rab7 and *T. denticola*. * means $p \leq 0.05$ between marked samples.

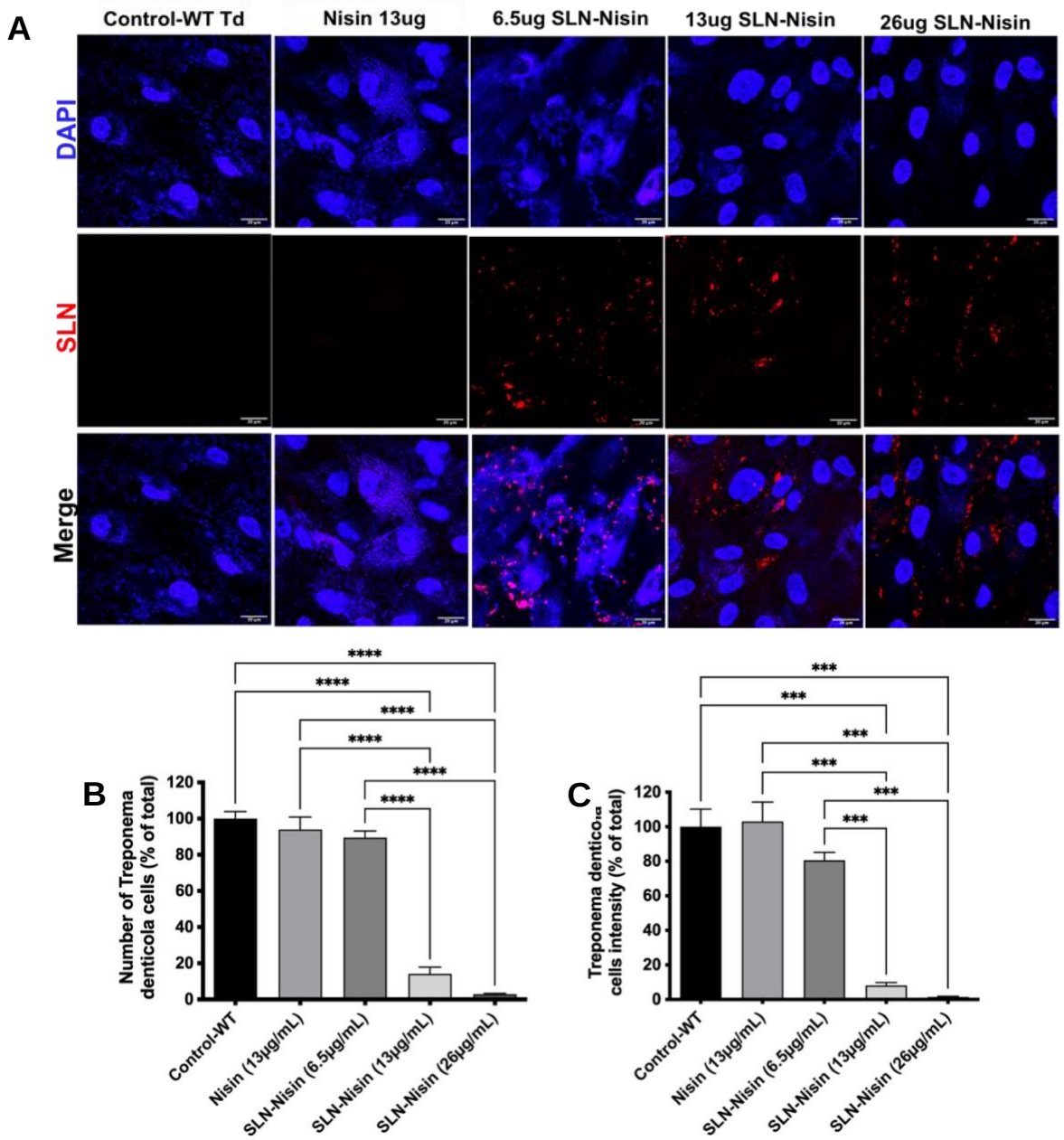


Figure 3.3.8. SLN-Nisin reduces intracellular and extracellular *T. denticola* after 48hrs. (A) Confocal microscopy of *T. denticola* genetic material (Top row; DAPI; Blue), SLN-Nisin (Middle row; Red) and the merged image (bottom row). (B) Percentage of positive hPDL cells for *T. denticola* genetic material in the images. (C) Percentage of fluorescence intensity relative to control (*T. denticola* infection) *** means $p \leq 0.001$ between marked samples and **** means $p \leq 0.0001$ between marked samples.

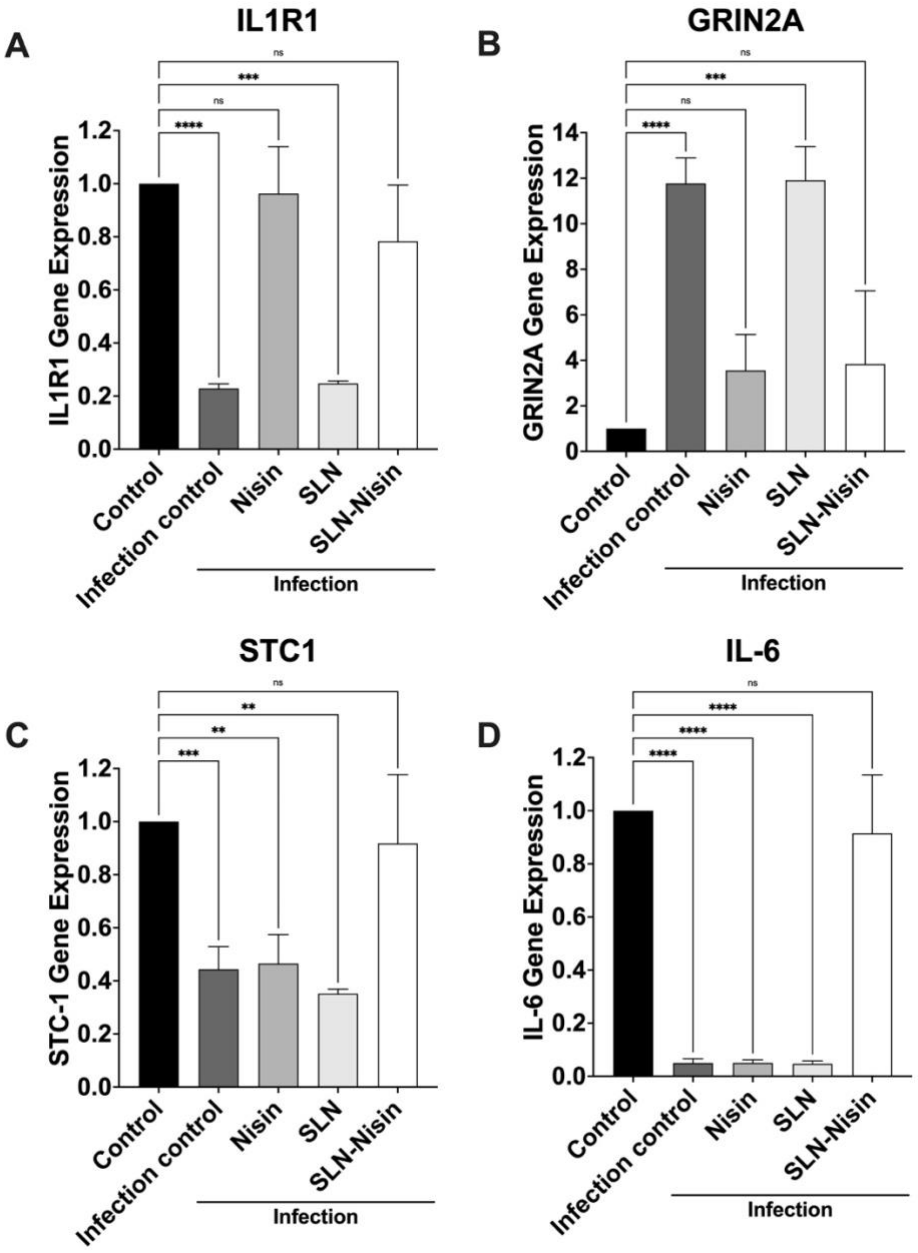
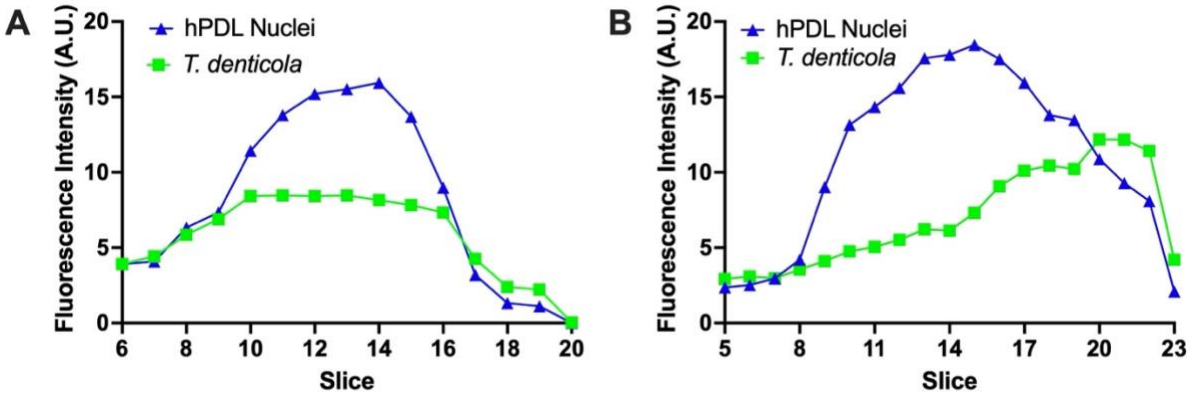
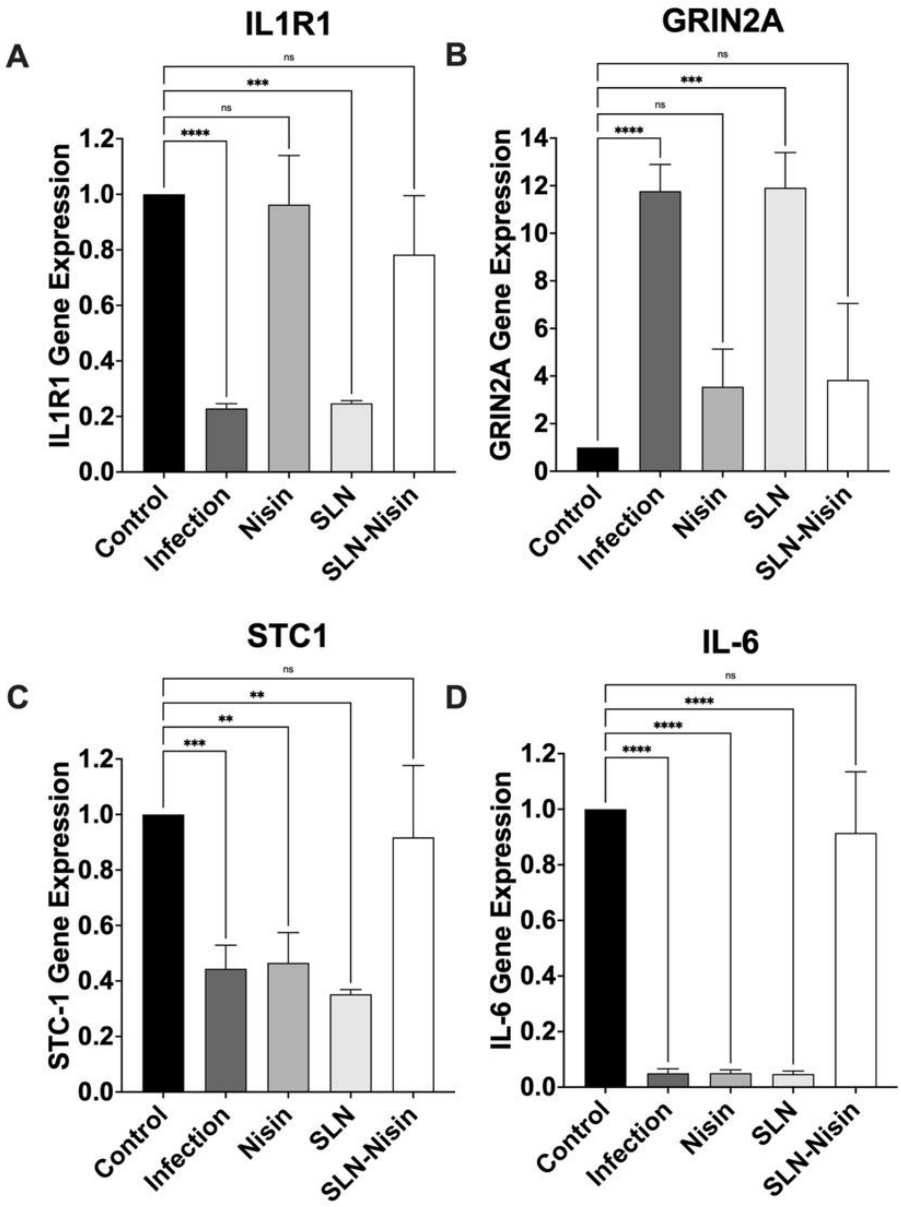


Figure 3.3.9. SLN-Nisin reduces *T. denticola* infection on IL1R1 (A), GRIN2A (B), STC1 (C) and IL-6 (D) gene expressions towards control levels. ** means $p \leq 0.01$ between marked samples, *** means $p \leq 0.001$ between marked samples and **** means $p \leq 0.0001$ between marked samples.

Chapter 3.7: Supplemental Figures



Supplemental Figure 3.7.1 *Treponema denticola* is localized inside of hPDL cells for up to 24h. (A) Fluorescence intensity of each slice of one Z-Stack for hPDL cell nuclei (Blue) and *T. denticola* anti-MSP antibody (green) after 12h of infection. (B) Fluorescence intensity of each slice of one Z-Stack for hPDL cell nuclei (Blue) and *T. denticola* anti-MSP antibody (green) after 24h of infection.



Supplemental Figure 3.7.2. SLN-Nisin reduces IL1R1 (A), GRIN2A (B), STC1 (C) and IL-6 (D) gene expressions of towards control (non-infected) levels. ** means $p \leq 0.01$ between marked samples, *** means $p \leq 0.001$ between marked samples and **** means $p \leq 0.0001$ between marked samples.

Chapter 3.8: REFERENCES

1. Ganther, S.; Radaic, A.; Malone, E.; Kamarajan, P.; Chang, N.-Y.N.; Tafolla, C.; Zhan, L.; Fenno, J.C.; Kapila, Y.L. *Treponema Denticola* Dentilisin Triggered TLR2/MyD88 Activation Upregulates a Tissue Destructive Program Involving MMPs via Sp1 in Human Oral Cells. *PLoS Pathog.* **2021**, *17*, e1009311, doi:10.1371/journal.ppat.1009311.
2. Malone, E.; Ganther, S.; Mena, N.; Radaic, A.; Shariati, K.; Kindberg, A.; Tafolla, C.; Kamarajan, P.; Fenno, J.; Zhan, L.; et al. *Treponema Denticola*-Induced RASA4 Upregulation Mediates Cytoskeletal Dysfunction and MMP-2 Activity in Periodontal Fibroblasts. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 671968, doi:10.3389/fcimb.2021.671968.
3. Radaic, A.; Kapila, Y. The Oralome and Its Dysbiosis: New Insights into Oral Microbiome-Host Interactions. *Comput. Struct. Biotechnol. J.* **2021**, *19*, 1335–1360, doi:10.1016/j.csbj.2021.02.010.
4. Eke, P.I.; Thornton-Evans, G.O.; Wei, L.; Borgnakke, W.S.; Dye, B.A.; Genco, R.J. Periodontitis in US Adults: National Health and Nutrition Examination Survey 2009-2014. *J. Am. Dent. Assoc. 1939* **2018**, *149*, 576-588.e6, doi:10.1016/j.adaj.2018.04.023.
5. Tonetti, M.S.; Jepsen, S.; Jin, L.; Otomo-Corgel, J. Impact of the Global Burden of Periodontal Diseases on Health, Nutrition and Wellbeing of Mankind: A Call for Global Action. *J Clin Periodontol* **2017**, *44*, 456–462, doi:10.1111/jcpe.12732.
6. Kumar, P.S. From Focal Sepsis to Periodontal Medicine: A Century of Exploring the Role of the Oral Microbiome in Systemic Disease. *J. Physiol.* **2017**, *595*, 465–476, doi:10.1113/JP272427.
7. Radaic, A.; Ganther, S.; Kamarajan, P.; Grandis, J.; Yom, S.S.; Kapila, Y.L. Paradigm Shift in the Pathogenesis and Treatment of Oral Cancer and Other Cancers Focused on the Oralome and Antimicrobial-Based Therapeutics. *Periodontol. 2000* **2021**, *87*, 76–93, doi:10.1111/prd.12388.
8. Ye, C.; You, M.; Huang, P.; Xia, Z.; Radaic, A.; Tang, J.; Wu, W.; Wu, Y.; Kapila, Y. Clinical Study Showing a Lower Abundance of Neisseria in the Oral Microbiome Aligns with Low Birth

Weight Pregnancy Outcomes. *Clin. Oral Investig.* **2022**, *26*, 2465–2478, doi:10.1007/s00784-021-04214-x.

9. Kamarajan, P.; Ateia, I.; Shin, J.M.; Fenno, J.C.; Le, C.; Zhan, L.; Chang, A.; Darveau, R.; Kapila, Y.L. Periodontal Pathogens Promote Cancer Aggressivity via TLR/MyD88 Triggered Activation of Integrin/FAK Signaling That Is Therapeutically Reversible by a Probiotic Bacteriocin. *PLoS Pathog.* **2020**, *16*, e1008881, doi:10.1371/journal.ppat.1008881.
10. Mombelli, A.; Samaranayake, L.P. Topical and Systemic Antibiotics in the Management of Periodontal Diseases. *Int. Dent. J.* **2004**, *54*, 3–14, doi:10.1111/j.1875-595X.2004.tb00246.x.
11. Bongers, S.; Hellebrekers, P.; Leenen, L.P.H.; Koenderman, L.; Hietbrink, F. Intracellular Penetration and Effects of Antibiotics on Staphylococcus Aureus Inside Human Neutrophils: A Comprehensive Review. *Antibiotics* **2019**, *8*, 54, doi:10.3390/antibiotics8020054.
12. Abed, N.; Couvreur, P. Nanocarriers for Antibiotics: A Promising Solution to Treat Intracellular Bacterial Infections. *Int. J. Antimicrob. Agents* **2014**, *43*, 485–496, doi:10.1016/j.ijantimicag.2014.02.009.
13. Aslam, B.; Wang, W.; Arshad, M.I.; Khurshid, M.; Muzammil, S.; Rasool, M.H.; Nisar, M.A.; Alvi, R.F.; Aslam, M.A.; Qamar, M.U.; et al. Antibiotic Resistance: A Rundown of a Global Crisis. *Infect. Drug Resist.* **2018**, *11*, 1645–1658, doi:10.2147/IDR.S173867.
14. Dutescu, I.A.; Hillier, S.A. Encouraging the Development of New Antibiotics: Are Financial Incentives the Right Way Forward? A Systematic Review and Case Study. *Infect. Drug Resist.* **2021**, *Volume 14*, 415–434, doi:10.2147/IDR.S287792.
15. World Health Organization *2019 Antibacterial Agents in Clinical Development: An Analysis of the Antibacterial Clinical Development Pipeline*; World Health Organization: Geneva, 2019; ISBN 978-92-4-000019-3.
16. Radaic, A.; de Jesus, M.B.; Kapila, Y.L. Bacterial Anti-Microbial Peptides and Nano-Sized Drug Delivery Systems: The State of the Art toward Improved Bacteriocins. *J. Controlled Release* **2020**, *321*, 100–118, doi:10.1016/j.jconrel.2020.02.001.

17. Radaic, A.; Ye, C.; Parks, B.; Gao, L.; Kuraji, R.; Malone, E.; Kamarajan, P.; Zhan, L.; Kapila, Y.L. Modulation of Pathogenic Oral Biofilms towards Health with Nisin Probiotic. *J. Oral Microbiol.* **2020**, *12*, 1809302, doi:10.1080/20002297.2020.1809302.
18. Shin, J.M.; Ateia, I.; Paulus, J.R.; Liu, H.; Fenno, J.C.; Rickard, A.H.; Kapila, Y.L. Antimicrobial Nisin Acts against Saliva Derived Multi-Species Biofilms without Cytotoxicity to Human Oral Cells. *Front. Microbiol.* **2015**, *6*, 617, doi:10.3389/fmicb.2015.00617.
19. Kajwadkar, R.; Shin, J.M.; Lin, G.-H.; Fenno, J.C.; Rickard, A.H.; Kapila, Y.L. High-Purity Nisin Alone or in Combination with Sodium Hypochlorite Is Effective against Planktonic and Biofilm Populations of Enterococcus Faecalis. *J. Endod.* **2017**, *43*, 989–994, doi:10.1016/j.joen.2017.01.034.
20. Gao, L.; Kuraji, R.; Zhang, M.J.; Martinez, A.; Radaic, A.; Kamarajan, P.; Le, C.; Zhan, L.; Ye, C.; Rangé, H.; et al. Nisin Probiotic Prevents Inflammatory Bone Loss While Promoting Reparative Proliferation and a Healthy Microbiome. *Npj Biofilms Microbiomes* **2022**, *8*, 45, doi:10.1038/s41522-022-00307-x.
21. Radaic, A.; Brody, H.; Contreras, F.; Hajfathalian, M.; Lucido, L.; Kamarajan, P.; Kapila, Y.L. Nisin and Nisin Probiotic Disrupt Oral Pathogenic Biofilms and Restore Their Microbiome Composition towards Healthy Control Levels in a Peri-Implantitis Setting. *Microorganisms* **2022**, *10*, 1336, doi:10.3390/microorganisms10071336.
22. Joo, N.E.; Ritchie, K.; Kamarajan, P.; Miao, D.; Kapila, Y.L. Nisin, an Apoptogenic Bacteriocin and Food Preservative, Attenuates HNSCC Tumorigenesis via CHAC1. *Cancer Med.* **2012**, *1*, 295–305, doi:10.1002/cam4.35.
23. Kamarajan, P.; Hayami, T.; Matte, B.; Liu, Y.; Danciu, T.; Ramamoorthy, A.; Worden, F.; Kapila, S.; Kapila, Y. Nisin ZP, a Bacteriocin and Food Preservative, Inhibits Head and Neck Cancer Tumorigenesis and Prolongs Survival. *Plos One* **2015**, *10*, e0131008, doi:10.1371/journal.pone.0131008.

24. Shin, J.M.; Gwak, J.W.; Kamarajan, P.; Fenno, J.C.; Rickard, A.H.; Kapila, Y.L. Biomedical Applications of Nisin. *J. Appl. Microbiol.* **2016**, *120*, 1449–1465, doi:10.1111/jam.13033.
25. Rojo, J.; Sousa-Herves, A.; Mascaraque, A. Perspectives of Carbohydrates in Drug Discovery. In *Comprehensive Medicinal Chemistry III*; Elsevier, 2017; pp. 577–610 ISBN 978-0-12-803201-5.
26. Patra, J.K.; Das, G.; Fraceto, L.F.; Campos, E.V.R.; Rodriguez-Torres, M. del P.; Acosta-Torres, L.S.; Diaz-Torres, L.A.; Grillo, R.; Swamy, M.K.; Sharma, S.; et al. Nano Based Drug Delivery Systems: Recent Developments and Future Prospects. *J. Nanobiotechnology* **2018**, *16*, 71, doi:10.1186/s12951-018-0392-8.
27. Radaic, A.; Barbosa, L.R.S.; Jaime, C.; Kapila, Y.L.; Pessine, F.B.T.; de Jesus, M.B. How Lipid Cores Affect Lipid Nanoparticles as Drug and Gene Delivery Systems. In; Advances in Biomembranes and Lipid Self-Assembly; Elsevier, 2016; Vol. 24, pp. 1–42 ISBN 978-0-12-804708-8.
28. Radaic, A.; de Jesus, M.B. Solid Lipid Nanoparticles Release DNA upon Endosomal Acidification in Human Embryonic Kidney Cells. *Nanotechnology* **2018**, *29*, 315102, doi:10.1088/1361-6528/aac447.
29. Radaic, A.; de Paula, E.; de Jesus, M.B. Factorial Design and Development of Solid Lipid Nanoparticles (SLN) for Gene Delivery. *J. Nanosci. Nanotechnol.* **2015**, *15*, 1793–1800, doi:10.1166/jnn.2015.9002.
30. Radaic, A.; Malone, E.; Kamarajan, P.; Kapila, Y.L. Solid Lipid Nanoparticles Loaded with Nisin (SLN-Nisin) Are More Effective Than Free Nisin as Antimicrobial, Antibiofilm, and Anticancer Agents. *J. Biomed. Nanotechnol.* **2022**, *18*, 1227–1235, doi:10.1166/jbn.2022.3314.
31. de Jesus, M.B.; Radaic, A.; Zuhorn, I.S.; de Paula, E. Microemulsion Extrusion Technique: A New Method to Produce Lipid Nanoparticles. *J. Nanoparticle Res. Interdiscip. Forum Nanoscale Sci. Technol.* **2013**, *15*, 1960, doi:10.1007/s11051-013-1960-3.

32. Mendonça, M.C.P.; Radaic, A.; Fossa, F.G.; Cruz-Höfling, M.A.; de Jesus, M.B. Toxicity of Cationic Solid Lipid Nanoparticles in Rats. *J. Phys. Conf. Ser.* **2019**, *1323*, 012016, doi:10.1088/1742-6596/1323/1/012016.
33. Ateia, I.M.; Sutthiboonyapan, P.; Kamarajan, P.; Jin, T.; Godovikova, V.; Kapila, Y.L.; Fenno, J.C. *Treponema Denticola* Increases MMP-2 Expression and Activation in the Periodontium via Reversible DNA and Histone Modifications. *Cell. Microbiol.* **2018**, *20*, doi:10.1111/cmi.12815.
34. Fenno, J.C. Laboratory Maintenance of *Treponema Denticola*. *Curr. Protoc. Microbiol.* **2006**, *Chapter 12*, Unit 12B.1, doi:10.1002/9780471729259.mc12b01s00.
35. Gamazo, C.; Prior, S.; Concepción Lecároz, M.; Vitas, A.I.; Campanero, M.A.; Pérez, G.; Gonzalez, D.; Blanco-Prieto, M.J. Biodegradable Gentamicin Delivery Systems for Parenteral Use for the Treatment of Intracellular Bacterial Infections. *Expert Opin. Drug Deliv.* **2007**, *4*, 677–688, doi:10.1517/17425247.4.6.677.
36. de Jesus, M.B.; Radaic, A.; Hinrichs, W.L.J.; Ferreira, C.V.; de Paula, E.; Hoekstra, D.; Zuhorn, I.S. Inclusion of the Helper Lipid Dioleoyl-Phosphatidylethanolamine in Solid Lipid Nanoparticles Inhibits Their Transfection Efficiency. *J. Biomed. Nanotechnol.* **2014**, *10*, 355–365, doi:10.1166/jbn.2014.1804.
37. Radaic, A.; Pugliese, G.O.; Campese, G.C.; Pessine, F.B.T.; de Jesus, M.B. [Studying the Interactions Between Nanoparticles and Biological Systems]. *Quím. Nova* **2016**, doi:10.21577/0100-4042.20160146.
38. Yamada, M.; Ikegami, A.; Kuramitsu, H.K. Synergistic Biofilm Formation by *Treponema Denticola* and Porphyromonas Gingivalis. *FEMS Microbiol. Lett.* **2005**, *250*, 271–277, doi:10.1016/j.femsle.2005.07.019.
39. Metsalu, T.; Vilo, J. ClustVis: A Web Tool for Visualizing Clustering of Multivariate Data Using Principal Component Analysis and Heatmap. *Nucleic Acids Res.* **2015**, *43*, W566–W570, doi:10.1093/nar/gkv468.

40. Skjeldal, F.M.; Haugen, L.H.; Mateus, D.; Frei, D.M.; Rødseth, A.V.; Hu, X.; Bakke, O. *De Novo* Formation of Early Endosomes during Rab5-to-Rab7a Transition. *J. Cell Sci.* **2021**, *134*, jcs254185, doi:10.1242/jcs.254185.
41. Vanlandingham, P.A.; Ceresa, B.P. Rab7 Regulates Late Endocytic Trafficking Downstream of Multivesicular Body Biogenesis and Cargo Sequestration. *J. Biol. Chem.* **2009**, *284*, 12110–12124, doi:10.1074/jbc.M809277200.
42. Cioni, J.-M.; Lin, J.Q.; Holtermann, A.V.; Koppers, M.; Jakobs, M.A.H.; Azizi, A.; Turner-Bridger, B.; Shigeoka, T.; Franze, K.; Harris, W.A.; et al. Late Endosomes Act as MRNA Translation Platforms and Sustain Mitochondria in Axons. *Cell* **2019**, *176*, 56-72.e15, doi:10.1016/j.cell.2018.11.030.
43. Kwon, T.; Lamster, I.B.; Levin, L. Current Concepts in the Management of Periodontitis. *Int. Dent. J.* **2021**, *71*, 462–476, doi:10.1111/idj.12630.
44. Inagaki, S.; Kimizuka, R.; Kokubu, E.; Saito, A.; Ishihara, K. *Treponema Denticola* Invasion into Human Gingival Epithelial Cells. *Microb. Pathog.* **2016**, *94*, 104–111, doi:10.1016/j.micpath.2016.01.010.
45. Shin, J.; Choi, Y. The Fate of *Treponema Denticola* within Human Gingival Epithelial Cells: *T. Denticola* Invasion of Epithelial Cell. *Mol. Oral Microbiol.* **2012**, *27*, 471–482, doi:10.1111/j.2041-1014.2012.00660.x.
46. Miao, D.; Godovikova, V.; Qian, X.; Seshadrinathan, S.; Kapila, Y.L.; Fenno, J.C. *Treponema Denticola* Upregulates MMP-2 Activation in Periodontal Ligament Cells: Interplay between Epigenetics and Periodontal Infection. *Arch. Oral Biol.* **2014**, *59*, 1056–1064, doi:10.1016/j.archoralbio.2014.06.003.
47. Baranov, M.V.; Kumar, M.; Sacanna, S.; Thutupalli, S.; van den Bogaart, G. Modulation of Immune Responses by Particle Size and Shape. *Front. Immunol.* **2021**, *11*, 607945, doi:10.3389/fimmu.2020.607945.

48. Paul, D.; Achouri, S.; Yoon, Y.-Z.; Herre, J.; Bryant, C.E.; Cicuta, P. Phagocytosis Dynamics Depends on Target Shape. *Biophys. J.* **2013**, *105*, 1143–1150, doi:10.1016/j.bpj.2013.07.036.
49. Champion, J.A.; Walker, A.; Mitragotri, S. Role of Particle Size in Phagocytosis of Polymeric Microspheres. *Pharm. Res.* **2008**, *25*, 1815–1821, doi:10.1007/s11095-008-9562-y.
50. Kou, L.; Sun, J.; Zhai, Y.; He, Z. The Endocytosis and Intracellular Fate of Nanomedicines: Implication for Rational Design. *Asian J. Pharm. Sci.* **2013**, *8*, 1–10, doi:10.1016/j.ajps.2013.07.001.
51. Bian X-L, Wang H-T, Ning Y, Lee SY, Fenno JC. 2005. Mutagenesis of a novel gene in the prcA-prtP protease locus affects expression of *Treponema denticola* membrane complexes. Infect Immun 73:1252–1255. doi: 10.1128/IAI.73.2.1252-1255.2005.

CHAPTER 4: CONCLUSION

Periodontal disease is a chronic inflammatory disease of the tooth supporting tissues that affect approximately 50% of the US adult population. The disease is driven by pathogenic microbes, including *Treponema denticola*, which is a key pathogen responsible for the breakdown of periodontal tissues. Among these tissues, the periodontal ligament is the key regenerative tissue of the periodontium. Therefore, *Treponema denticola* interactions with the periodontal ligament are of particular interest in understanding the pathogenesis of the disease. Our study is one of the first to investigate interactions on cytopathic functions needed for the PDL to maintain homeostasis. Under our *Td* infection model, we highlighted PDL cell increase in metabolic activity, migration during wound healing, while no effect was significantly shown to decrease contractility of collagen type I gels. An underpinning cellular system connecting these functions is the actin cytoskeletal process which led us to investigate a potential disruption in actin dynamics. We highlighted a novel mechanism by which *Treponema denticola* triggers enhanced RASA4 gene expression in periodontal ligament cells as part of the tissue breakdown process that involves changes in actin fiber re-organization and MMP-2 expression. This study provides foundational new knowledge that the pathogenesis of periodontal disease. Along with this novel mechanism, we demonstrate that it is mediate through the virulent protein and protease, dentilisin.
Dentilisin is a key serine protease from *Treponema denticola* that was shown in this study to effectively activate the inactive pro-from of MMP-2 directly. Together these findings provide more evidence of *Td* as a dual threat for ECM destruction through 1) direct interactions with MMPs and 2) cytopathic functions that enhance MMP production and activation at a protein level and transcriptionally.

While elucidating adverse effects of periodontal pathogens are of the utmost importance, there are still needs to combat these bacteria. The final aspect of our study looks into developing a promising bacteriocin and drug delivery system that can revolutionize treatment of periodontal disease. We decided to focus on *Td* as the target of choice. In order to properly know how to target *Td*, we followed its internalization mechanism to enter hPDL cells. From our investigation, we concluded that *T. denticola*

and our drug delivery system SLN-Nisin enter primary human PDL cells via endocytosis. Our studies suggest *T. denticola,* like periodontopathogen *P. gingivalis,* should be viewed as an intracellular bacterium in the context of periodontal disease. Additionally, SLN-Nisin can aid in clearance of intracellular pathogens, like T*. denticola*. Thus, making SLN-Nisin complex an innovative way to improve antibiotics and bacteriocins used to target both intracellular and extracellular pathogens of periodontal disease and other bacterial-induced diseases.

Our study is the first to show nisin targeting intracellular pathogens. We further showed SLN-Nisin to reverse immunocompromised genetic response of hPDL cells due to *Td* infection. Therefore, nisin has hopeful capability to inhibit periodontopathogen bacteria inside and outside tissues, diminish immune response to dysbiotic biofilms, and potentially reset infected periodontium tissues to healthy status. Our study provides a foundation for SLN-Nisin as a potential antimicrobial treatment therapy. However more studies are necessary to move SLN-Nisin from in vitro studies into clinical trial experiments.

Publishing Agreement

It is the policy of the University to encourage open access and broad distribution of all theses, dissertations, and manuscripts. The Graduate Division will facilitate the distribution of UCSF theses, dissertations, and manuscripts to the UCSF Library for open access and distribution. UCSF will make such theses, dissertations, and manuscripts accessible to the public and will take reasonable steps to preserve these works in perpetuity.

I hereby grant the non-exclusive, perpetual right to The Regents of the University of California to reproduce, publicly display, distribute, preserve, and publish copies of my thesis, dissertation, or manuscript in any form or media, now existing or later derived, including access online for teaching, research, and public service purposes.

DocuSigned by:
F5A8C61F8B524E8...
Author Signature

8/14/2023
Date