Bacterial Internalization During Human Experimental Gingivitis: A Quasi-Longitudinal Study

by
Anjali Bhagirath

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Medical Sciences – Periodontology
University of Alberta

ABSTRACT

Introduction: Bacterial invasion from subgingival plaque into gingival tissues is a critical aspect of periodontal disease pathogenesis. This invasion not only contributes to disease progression but also enables the pathogens to survive within the host and evade immune surveillance. While previous studies have identified some bacteria that penetrate host tissues in advanced periodontitis, the full spectrum in early gingivitis is not known. Our research breaks new ground by mapping out the bacteria originating from dental plaque that are capable of infiltrating host gingival tissues by leveraging a modification of the human experimental gingivitis model.

Materials and Methods: In our study (HREB: Pro00112019), 22 healthy participants underwent a three-phase Human Experimental Gingivitis investigation: baseline, induction, and resolution. Baseline was set post-professional cleaning before the start of the study. Using a partial-mouth approach with an acrylic stent, we induced gingivitis in randomly chosen mouth quadrants by pausing oral hygiene for up to 21 days, with the other quadrant as controls. This comprised the induction phase. Post-induction, regular hygiene was re-instituted and gingival health was restored. Clinical parameters with standard Indices such as PPD, BOP, PI, MQHPI, and PMA were recorded. Samples of subgingival plaque and gingival biopsies cleared of extracellular bacteria using gentamycin, underwent 16S rDNA sequencing for bacterial identification. The data were analyzed using systems biology approach to assess microbial taxonomy and functionality.

Results: Over the 21-days PPD, BOP, PI, and MQHPI increased significantly ($p<0.05$). The microbiome analysis reinforced the 'nepotism hypothesis', indicating non-random clustering of plaque microbes ($p<0.05$). Plaque showed higher microbial richness yet less uniformity in species distribution ($p<0.05$). Both plaque and tissues saw a decline in Shannon diversity without an increase in strain numbers, suggesting selective microbial persistence ($p<0.05$, Wilcoxon signed

rank sum test). Plaque had more core taxa compared to tissues, and over time, beta-diversity within tissues diminished, denoting a more limited microbial community ($p<0.05$, FDR post-hoc of Friedman's test on PhILR distances). Specific plaque microbes significantly affected the diversity of gingival tissue taxa ($p<0.05$, extended local similarity analysis). Other taxa modified these effects ($p<0.05$, liquid association) indicating complex, non-linear inter-bacterial interactions.

Conclusions: Our findings reveal that variations in plaque and gingival microbiome are driven by shifts within existing microbial communities, rather than introductions of new species. Gingival tissues exhibit less diversity compared to plaque, indicating a selective environment that favors specific taxa early in the inflammation process. This highlights the pivotal role of microbial selection in shaping the progression of gingival inflammation.

PREFACE

This thesis is an original work by Anjali Bhagirath. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “GINGIVITIS 21”, No. Pro00112019, obtained: Sep. 2021.

DEDICATION

I would like to dedicate this thesis to Juno, Peaches and Saurabh

ACKNOWLEDGEMENTS

In the swashbuckling saga that is research, I must salute my supervisor a.k.a Captain Dr. Khaled Altabtbaei for not making me walk the plank in the experimental gales, and Dr. Monica Gibson, who prevented us from capsizing in the whirlpool of confusions.

Dr. Khaled's mentorship has been the compass that navigated me through the wilderness of this research, and his patience has been the anchor in the often-stormy seas of my learning curve. I thank you for your belief in my potential.

To my lab comrades, particularly our sun-chasing summer students Brianne, Johanna, my brother Ramon and Lavanya, your camaraderie and resilience are cornerstones of our lab. Special applause to Dr. Maria Alexiou, whose support kept our lab well-stocked and only marginally less chaotic than a toddler's birthday party. And let's not forget the mysterious case of the randomly appearing and disappearing coffee cups—your silence on this matter has been noted and is much appreciated!!

Thanks to Drs. Febbraio and Graf, whose feedback was the lighthouse guiding us through the foggy waters of wet-lab experimentation. Hats off to the University of Alberta for their financial support—it was nothing short of a fiscal ballet, and we danced to the tune of science. To the administrative maestros at the Kaye Edmonton Clinic, Periodontology, especially Shirlene, Teena, Sandra and Cheryl, who navigated my last-minute antics with the grace of swans, you have my eternal thanks.

In the lab that Dr. Khaled helms, I've found more than just colleagues; I've found a second family. To my academic siblings, Dr. Andrew Gibb and Dr. Anum Haider, I thank you for the shared shivers and snackless afternoons—your solidarity was the secret ingredient in our lab's elixir of success.

Finally, a stealthy nod to Dr. Dederich, who allowed me to tiptoe between clinical duties and clandestine lab work. Your leniency was the covert operation behind many a breakthrough.

A thunderous round of applause to my comrades-in-arms, the venerable Drs. Huma, Crystal, Nisha, Jane S., D, Z and my ‘T’. You've been the co-authors in the often-spiraling draft of my life and the sometimes-chaotic narrative of my academic journey. Regrettably, you were conscripted into this saga with little say. For that, I offer a bounty of thanks. In the grand finale of my acknowledgments, a whimsical tip of the hat to Manoj, who has bravely weathered the storm of my many stupid mistakes and eccentricities as my partner in most-things-life. And to Vasu, the

guardian of my title as ‘Numero Uno Best Friend'—thank you both for taking in the menagerie of loveable critters that I conveniently 'foster-fail'. Your endorsement for future raccoon parenthood is eagerly awaited. A tip of the mortarboard to Drs. Cholakis, Gilchrist, Dakshinamurti, Glogauer and Chelikani, for everything. Your guidance has been the secret sauce in my academic journey!

To my beloved fur children—Sparrow, Jack, Charlie, and Remy—and the dear departed who've crossed the rainbow bridge, you've given me a taste of motherhood. You've graced my life with purrs, paws, and unconditional love, and for that, you have my eternal gratitude.

As the curtain falls on this academic performance, a thunderous ovation is due for my family—my stalwart parents, my sisters, brother, and the illustrious Manoj contingent. You've all stood by us through countless hours of scholarly pursuits that, to the untrained eye, might resemble the mysterious rituals of a secret society. Yes, life is indeed an anthology of puzzling choices, and your fortitude in the face of this is nothing less than legendary.

And for those who've slipped through the cracks of my memory—take a bow. Your support has been the secret ingredient in this concoction of education and roaring insanity. I offer you a namaste from the bottom of my heart.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

ASV: Amplicon Sequence Variant

BOP: Bleeding on Probing

CLRs: Centered Log-Ratio

DNA: Deoxyribonucleic Acid

eLSA: Extended Local Similarity Analysis

ENAP: Excision New Attachment Procedure

FDR: False Discovery Rate

GCF: Gingival Crevicular Fluid

HOMD: Human Oral Microbiome Database

PhILR: Phylogenetic Isometric Log-Ratio

MQHPI: Modified Quigley Hein Plaque Index

NCDs: Non-Communicable Diseases

OTU: Operational Taxonomic Unit

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PMA: Papillary Marginal Attachment

PPD: Probing Pocket Depth

rDNA: Ribosomal DNA

RNA: Ribonucleic Acid

SD: Standard Deviation

TSA-NAM: Tryptic Soy Agar with N-Acetylmuramic Acid

CHAPTER 1: INTRODUCTION

1.1. Epidemiology of Periodontal diseases

Oral diseases have exceeded the global prevalence of all other non-communicable diseases (NCDs). Dental caries and periodontitis are the most prevalent oral conditions, affecting approximately 2 billion and 1 billion individuals worldwide, respectively (WHO, 2022). Periodontal diseases are conditions that involve both soft and hard tissues around teeth. It encompasses conditions such as gingivitis, a reversible form of gingival inflammation marked by swollen and bleeding gums with no permanent loss of hard and soft tissue structures around teeth. As per the recent joint EFP/AAP workshop, gingivitis is defined as the presence of ≥ 10% of sites that bleed upon probing along with clinical signs of inflammation without progressive loss of supporting hard tissues. Gingivitis is the most common form of oral disease in children and adolescents (Albandar & Rams, 2002; Clerehugh, 2008; Oh, Eber, & Wang, 2002). Epidemiologically, Albandar and Kingman estimated gingivitis to affect more than 50% of the adults in the United States (U.S.)(Albandar & Kingman, 1999). More recently, Petersen & Ogawa estimated the global prevalence of gingivitis in adults to range from 10% to 15% (Petersen & Ogawa, 2012). As gingivitis is clinically undetectable in its early stages, the reported prevalence might likely be an underestimation (Scannapieco, 2004).

The continued progression of gingival inflammation results in a more chronic and irreversible form known as Periodontitis. The burden of periodontal disease has been increasing, with studies reporting a 57.3% rise in the global burden of periodontal disease from 1990 to 2010 (Nazir et al., 2020; Tonetti, Jepsen, Jin, & Otomo-Corgel, 2017). Furthermore, it has been estimated that severe periodontitis affects approximately 743 million people worldwide, making it the sixth most prevalent disease globally (Arias-Bujanda et al., 2019; Tonetti et al., 2017). The global prevalence of periodontitis is predicted to increase in the coming years due to an increase in the aging population and lifestyle factors (WHO, 2022). It must be noted that the reported epidemiological numbers might be underrepresented as the studies are limited by factors such as sampling methods, technical factors such as diagnostic techniques, applied definitions and the examined populations and their cultural and socio-economic factors. However, it is agreed that periodontal diseases result in a significant socioeconomic burden. In 2010 alone, the worldwide loss of productivity due to severe periodontitis alone was estimated to be US $54 billion (Listl, Galloway, Mossey, & Marcenes, 2015). This is projected to be much larger when viewed globally. Thus, there is an emergent need to devise strategies and inform research in the field to effectively prevent and manage these diseases.

1.2. Pathogenesis of Gingivitis and Periodontitis

Periodontal health is defined broadly as the absence of clinically detectable inflammation (Chapple et al., 2018). The pathogenesis and development of gingivitis and periodontitis are multifactorial and involve various biological, environmental, and behavioural factors; however, bacterial plaque accumulation is a key factor, with specific species potentially playing a role in the progression of the disease. Without disruption or removal, accumulated dental plaque causes a loss of symbiosis between the host's inflammatory response and results in the development of an incipient dysbiosis, manifesting as clinical gingivitis (Lang & Bartold, 2018). Key indicators of gingivitis encompass observable clinical inflammation symptoms localized to the free and attached gingival areas, remaining within the boundaries of the mucogingival junction. This inflammation can typically be reversed by disrupting or removing the dental biofilm. A significant accumulation of bacterial plaque is necessary to trigger or intensify the severity of gingivitis, with the degree of impact differing from person to person(Kinane & Attström, 2005).

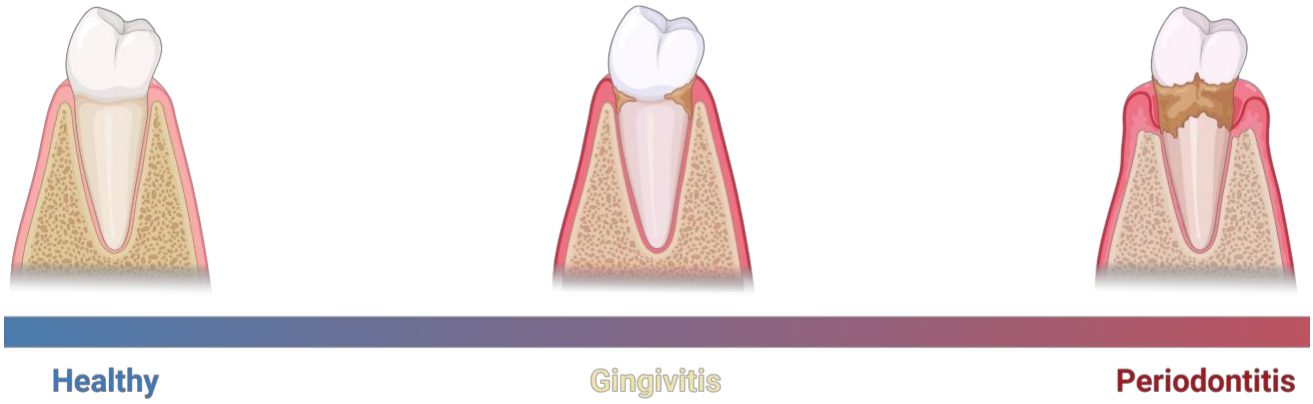

Figure 1:Progression from gingival health to periodontitis. Periodontal health is defined as the absence of clinically detectable inflammation. Without disruption or removal accumulated dental plaque causes inflammation of soft tissues around teeth resulting in clinical gingival inflammation. Further uninterrupted progression of gingivitis results in Periodontitis marked by loss of both hard and soft tissues around teeth.

1.2.1. Gingivitis and Subgingival Plaque Microbiome

The gingival sulcus presents as a distinct anatomical feature, flanked by the tooth on one side and soft tissue on the other. The interface between gingiva and tooth can harbour as many as 700 different bacterial species (Haffajee, Patel, & Socransky, 2008). While many of these bacteria live in symbiosis with the host organism, specific bacterial strains have demonstrated the capacity to destabilize the equilibrium between the bacteria and the host, leading to disease initiation and progression in combination with other factors.

Studies examining the subgingival microbiome have undergone significant advances over the past decade. The microbiological changes associated with the development of gingivitis are influenced by factors such as hormonal changes (Mombelli, Lang, Bürgin, & Gusberti, 1990) and the placement of subgingival restorations (Lang, Kiel, & Anderhalden, 1983). Mombelli (1990) found that the presence of *Capnocytophaga sp*. and black-pigmented Bacteroides, particularly *B. intermedius*, was associated with initiating and establishing puberty aasociated gingivitis (Mombelli et al., 1990). Other studies have reported increased levels of certain species, including *Eikenella corrodens, Fusobacterium nucleatum,* and *Capnocytophaga gingivalis,* in the subgingival plaque microbiome in human gingivitis (Savitt & Socransky, 1984). This shift in the microbiome is further evidenced by the presence of Gram-positive and Gram-negative species such as *Haemophilus parainfluenzae* and *Veillonella species* (Slots, Möenbo, Langebaek, & Frandsen, 1978). The microbiome in gingivitis is further characterized by an increase in obligate anaerobes, particularly *T. forsythia, P. gingivalis,* and *Eubacterium saphenum*, and a decrease in *Streptococcus species* (Abiko, Sato, Mayanagi, & Takahashi, 2010).

More recent studies on changes in the subgingival microbiome in gingivitis by enrichment of specific bacterial species, including *TM7, Tannerella, Cardiobacterium, Campylobacter, Porphyromonas, Leptotrichia*, and *Selenomonas* (Al-Kamel et al., 2019). The transition from health to gingivitis is marked by shifts in the abundance of several genera, indicating dysbiosis (Nowicki et al., 2018). These findings suggest that the subgingival microbiome in gingivitis is dynamic and influenced by various factors.

1.2.2. A Brief History of Research on Periodontal Microbiome, and Host Inflammatory Responses

The 1918 investigation by Turner and Drew found bacteria in "pyorrhea" lesions (Turner & Drew, 1919). These findings were confirmed by Beckwith et al., who showed bacteria invading inflamed gingival tissues in the histological section (Beckwith, Simonton, & Williams, 1925). Saglie et at. reported bacteria invading the epithelial wall and that bacterial invasion of the soft periodontal tissues was indeed a common finding in advanced periodontitis (Manor, Lebendiger, Shiffer, & Tovel, 1984; R. Saglie & Elbaz, 1983; R. Saglie, Newman, Carranza, & Pattison, 1982). These studies showed specific bacterial species, including cocci, rods, filaments, and spirochetes, invaded the gingival tissues in advanced periodontitis using light and scanning electron microscopy-based techniques. As the specific-plaque hypothesis theory was more widely accepted, associating specific species with certain periodontitis phenotypes was then widely attempted. In the seminal work of Christersson, it was shown that *Actinobacillus* (currently *Aggregatibacter*) *actinomycetemcomitans,* a pathogen mostly found in a rapidly progressing phenotype of periodontitis previously known as aggressive periodontitis, internalizes into the gingival tissues, and recolonizes the subgingival space after non-surgical therapy, leading to the notion that gingival tissues act as a reservoir for A. *actinomycetemcomitans* thereby needing supplemental antibiotics to remove this pathogen. However, bacterial internalization also occurs in the state of health. Periodontal pathogens, such as *P. gingivalis, Tannerella forsythia, A. actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia, Eikenella corrodens, and Treponema denticola* have been demonstrated in buccal and gingival epithelial cells of healthy individuals using Fluorescence in situ hybridization and confocal microscopy(A. Colombo, Da Silva, Haffajee, & Colombo, 2007; Rudney, Chen, & Sedgewick, 2005; Rudney, Chen, & Zhang, 2005).

<u>Studies in Non-Human Models</u>

Monkeys: Studies in non-human animal models have provided important foundational insights into plaque microbiome dynamics, bacterial invasion, and pathogenesis mechanisms in periodontal diseases (Schou, Holmstrup, & Kornman, 1993). Non-human primates, particularly monkeys, have been extensively utilized in periodontal disease research due to their anatomical and physiological similarities to humans. Studies have demonstrated that non-human primates exhibit clinical, microbiological, and immunological features of periodontal disease that closely resemble those observed in humans (A. P. V. Colombo et al., 2017; Oz & Puleo, 2011). Studies in the ligature-

induced periodontitis model of nonhuman primate *Macaca Mulatta* showed that the subgingival plaque microbiome interacts with gingival tissues to upregulate gene expression patterns associated with apoptosis, autophagy, and hypoxia pathways in progressing periodontitis, providing insights into the biological activities occurring in diseased gingival tissues(Ebersole, Kirakodu, Chen, Nagarajan, & González, 2020; Ebersole, Kirakodu, & González, 2021). Additionally, the acquisition of oral microbes in newborn non-human primates has been linked to systemic immune responses, indicating a relationship between microbiological and immunological studies of gingivitis and periodontitis in humans and non-human primates(Ebersole, Holt, & Delaney, 2013). While "close-enough" studies on nonhuman primates provide valuable models of disease, there is a variability in responses among different species of non-human primates can complicate the interpretation and generalization of research findings(Holt, Ebersole, Felton, Brunsvold, & Kornman, 1988; Weinberg & Bral, 1999).

Miniature Pigs/Dogs: Other animal models, such as miniature pigs and dogs, demonstrating structural similarities in periodontal organization to humans, have also been used in research. These have reported a distinct subgingival microbiome comprising mainly gram-negative species(Egelberg, 1965; Pavlica, Petelin, Nemec, Erzen, & Skaleric, 2004; Vodička et al., 2005; Wang, Liu, Fang, & Shi, 2007). Furthermore, the responses in both these species are dependent on genetic variations(Kwon et al., 2010).

Rodents: Some other animal models include rodents such as mice and rats. While these models make in vivo mechanistic explorations into disease pathogenesis feasible, they are limited due to several factors. Mice are naturally resistant to periodontitis. Furthermore, mice and humans' microbiota and immune responses differ significantly, making extrapolation difficult(Kesavalu et al., 2007; Marchesan et al., 2018; Rojas et al., 2021).

Humanized Animals: Early results from studies using humanized animals have reported a microbial profile distinct from the human oral cavity, limiting their use and extrapolation of findings to human subjects(Mason, 2019).

Building upon foundational insights from non-human animal models, such as monkeys, miniature pigs, dogs, and rodents, studies have elucidated key mechanisms underlying microbial invasion in periodontal diseases. These models, characterized by their structural and physiological similarities to humans, have provided critical insights into the interaction dynamics of subgingival microbiota with gingival tissues. Key limitations of animal models include differences in oral microbiome composition compared to humans and difficulty replicating the complexity of naturally occurring periodontal disease. Further intrinsic differences in parameters such as bone turnover rate, bony architecture, thickness, and density, as well as the genetic factors involved in periodontal disease progression, add to the complexity of extrapolating findings in these models(Giannobile, Finkelman, & Lynch, 1994; Kajikawa et al., 2017).

Nevertheless, these models remain invaluable for mechanistic explorations, paving the way for focused research in human experimental gingivitis studies.

1.3 Pathogenesis of Periodontal Disease and Microbial Invasion

It has been recognized for decades that there are two general mechanisms for pathogenesis, one involves the direct invasion of the microbiota into the gingival tissues, and second, whereby, according to Socransky et al. (Socransky & Haffajee, 1991), a *"long range"* attack occurs, when cells of pathogenic species remain in the periodontal pocket, and the released virulence factors from these pathogens can cause damage to host tissues as well as cause slow but gradual *"immune pathology"*. While evidence exists for both methods exists, knowing the importance and inter-play between both is important and is a critical avenue for future research. Regardless of type of microbial influence, the existence and the influence of the microbiome is based on the host response to their existence and actions **(Figure 2)**.

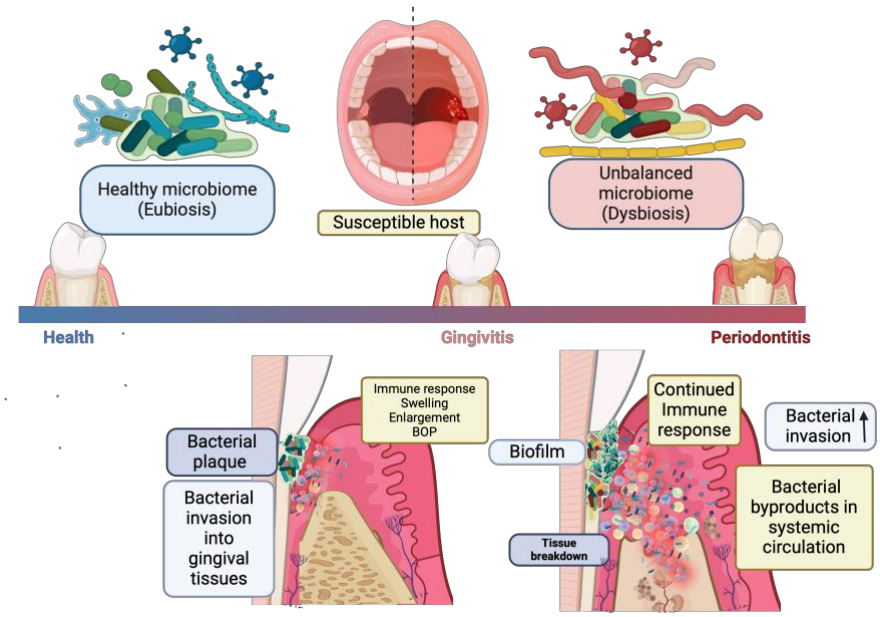

Figure 2: Microbial Invasion in Health and Disease. This figure illustrates the progression of microbial invasion and its impact on oral health, depicting the transition from a healthy microbiome (eubiosis) to an unbalanced microbiome (dysbiosis), and subsequently to gingivitis and periodontitis. The top panel depicts a balanced microbiome in a healthy state transitioning to dysbiosis in a susceptible host. The bottom panel shows the stages of oral health: from health, where the gingiva is intact, to gingivitis, characterized by bacterial plaque, invasion, and immune response (swelling, enlargement, BOP), and finally to periodontitis, marked by biofilm formation, tissue breakdown, and systemic bacterial byproducts. The figure was created using BioRender.com.

Invasion of pathogens plays a role for both the host and the pathogen. The host mucosal immunity responds to surface bacteria by antigen processing and continued "learning" and building a robust inflammatory response. For the invading pathogens, the host tissues offer a viable niche by evading clearance by circulating immune cells and providing residence, as well as a reservoir for re-infection (Rudney, Chen, & Zhang, 2005) and pathways for dissemination. Additionally, studies have shown that persisting internalized bacteria can mediate epigenetic changes within the host, leading to lasting long-term effects (Francis et al., 2020; Martins et al., 2016). As such, the capacity to infiltrate and remain within host cells plays a significant role in the progression of the disease.

Periodontal Clinical Phenotypes and Tissue Invasion: Early studies using transmission electron microscopy have revealed the presence of spirochetes and other microorganisms within the gingival epithelium and connective tissues, particularly in patients with acute necrotizing gingivitis (Courtois, Cobb, & Killoy, 1983; Listgarten, 1965). Studies, such as the one by Christersson et al., utilized immunofluorescence to detect *A. actinomycetemcomitans* in aggressive periodontitis cases, with further researches employing PCR, DNA hybridization, and FISH to explore intracellular invasion by this bacterium (Christersson, Albini, Zambon, Wikesjö, & Genco, 1987). Moreover, bacterial invasion has been considered a later event in periodontitis. Consequently, most periodontal microbiome studies have focused on characterizing plaque biofilms, excluding gingival tissues, despite the similarity in the microbial profiles between the two. Limited data on the internalization of subgingival biofilms warrants further investigation.

Microbial Actions During Tissue-Invasive Periods: Several studies demonstrated the internalization of bacteria such as *P. gingivalis, A. actinomycetemcomitans, T. denticola,* and *F. nucleatum* in human gingival tissues using culture-dependent methods such as *in vitro* and *in vivo* models of periodontitis (Christersson et al., 1987; P. Fives-Taylor, Meyer, & Mintz, 1995; R. Saglie et al., 1982; Saito, Inagaki, & Ishihara, 2009; Sandros, Papapanou, & Dahlén, 1993). Notably, one study found bacteria forming biofilm-like structures within gingival tissues in established periodontitis (Baek, Ji, & Choi, 2018). Additionally, 16s rRNA profiling showed that *F. nucleatum* and *P. gingivalis* were significantly enriched in tissues compared to plaque (Baek et al., 2018). The ability of *A. actinomycetemcomitans* to disrupt epithelial barrier function, induce apoptosis in lymphocytes, and evade host defenses by surviving within fibroblasts underscores its pathogenic

potential (P. Fives-Taylor et al., 1995; P. M. Fives-Taylor, Meyer, Mintz, & Brissette, 1999; Sreenivasan, Meyer, & Fives-Taylor, 1993). Similarly, *P. gingivalis* has been shown to invade various gingival cell types, manipulating host cell structures to facilitate its uptake and survival, highlighting the sophisticated mechanisms these bacteria employ to persist within their hosts(Belton, Izutsu, Goodwin, Park, & Lamont, 1999; Gibson III, Yumoto, Takahashi, Chou, & Genco, 2006; Lamont et al., 1995; Sandros et al., 1993; Sandros, Papapanou, Nannmark, & Dahlén, 1994; Ö. Yilmaz, Watanabe, & Lamont, 2002; O. Yilmaz, Young, Lamont, & Kenny, 2003).

Microbial Identification in Oral Tissues: Fredricks et al. found *Capnocytophaga sputigena, Leptotrichia spp*., and *F. nucleatum* in an immunocompromised patient (Fredricks, Schubert, & Myerson, 2005). Adrians et al., and Thiha et al., identified *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia* in similar contexts (Andrian, Grenier, & Rouabhia, 2004; Thiha et al., 2007). These discoveries, alongside the work of Saglie and Papapanou, link the presence of these bacteria to gingival inflammation and tissue destruction, underlining their significance in periodontal disease progression (Papapanou et al., 2009; F. R. Saglie et al., 1987; R. Saglie & Elbaz, 1983; R. Saglie et al., 1982). While most of the earlier research focused on perio-pathogens like *P. gingivalis* and *A. actinomycetemcomitans,* bacteria such as F. *nucleatum* and *T. forsythia*, have also been shown to invade host cells and manipulate immune responses(Bachrach, Ianculovici, Naor, & Weiss, 2005; Bao et al., 2014; Han et al., 2000; Inagaki, Kuramitsu, & Sharma, 2005; Inagaki, Onishi, Kuramitsu, & Sharma, 2006). Studies by Rudney et al. and others demonstrate the intracellular presence of a diverse microbial community within buccal cells (Rudney, Chen, & Sedgewick, 2005; Rudney, Chen, & Zhang, 2005), indicating a complex interaction network that influences disease progression.

Microbial Synergy: Co-infection studies have shown that *P. gingivalis* and *T. denticola* exhibit metabolic symbiosis and enhance each other's invasion and virulence. Conversely, *F. nucleatum* can promote *P. gingivalis* invasion, while *A. actinomycetemcomitans* and *T. forsythia* may inhibit it (Johansson, Hänström, & Kalfas, 2000; Jung, Jun, & Choi, 2017; Lima et al., 2002; Orth, O'Brien-Simpson, Dashper, & Reynolds, 2011; Saito et al., 2009; Tan et al., 2014). Such interactions are observed between pathogenic species and other species, such as those observed between *F. nucleatum* and *Streptococcus cristatus,* where *F. nucleatum* was shown to facilitate the

internalization of the latter into the host cells (Edwards, Grossman, & Rudney, 2006). In another study, *A. actinomycetemcomitans* was shown to inhibit *Candida albicans* biofilm formation (Bachtiar et al., 2014). These interactions, reveal a dynamic ecosystem within gingival tissues that affects disease outcome.

Acknowledging the limitations of single-species studies, recent research endeavours aim to explore multi-species invasions using cutting-edge techniques like multi-colour imaging and metagenomics. This shift towards investigating polymicrobial interactions is crucial for comprehending periodontal disease pathogenesis. It accurately reflects the in vivo conditions and potentially unveils new therapeutic targets to combat periodontal diseases more effectively. By embracing the complexity of these microbial communities, we can unlock novel insights into their collective impact on periodontal health and disease progression.

1.4. The Human Experimental Gingivitis Study

Experimental gingivitis is a widely used model to study the onset and progression of gingival inflammation in humans. This model, which induces gingivitis by allowing the accumulation of dental plaque, has enabled researchers to observe and understand the relationship between bacterial deposits, gingival inflammation, and the development of periodontal diseases. It also allows studying the full breadth of microbial complexity in humans in a non-invasive manner.

1.4.1 History

Studies on experimental gingivitis in humans have been conducted for several decades, with some of the earliest works dating back to the 1960s. Löe et al. (1965) performed a groundbreaking study investigating the relationship between plaque accumulation, gingival inflammation, and the histologic appearance of gingival lesions (Loe, Theilade, & Jensen, 1965). This study laid the foundation for future investigations into the microbiological aspects of experimental gingivitis, such as the work done by Theilade in 1996 (Theilade, Wright, Jensen, & Löe, 1966) . Over the years, numerous studies have been conducted using the experimental gingivitis model, leading to a better understanding of the pathogenesis of gingivitis and periodontitis.

1.4.2 Methodology and Modifications to the Classical Experiment

The classic human experimental gingivitis study methodology involves inducing and resolving gingivitis in a controlled manner to understand the pathogenesis and resolution of inflammation. The study typically comprises of several phases, including a pre-induction period, an induction period, and a resolution period. During pre-induction, participants undergo professional tooth cleaning, affirming a state of health and maintain their usual oral hygiene practices. Subsequently, the induction period involves the accumulation of dental plaque, often achieved by the cessation of oral hygiene practices for 21 days. This phase allows for the development of gingivitis under controlled conditions. The resolution period follows for a week after the Induction period, during which professional oral hygiene is performed, and participants resume regular oral hygiene practices, leading to the resolution of gingival inflammation (Bamashmous et al., 2021; Loe et al., 1965; Seymour, Powell, & Aitken, 1983; Theilade, 1996; Theilade et al., 1966).

The study methodology also involves bacteriological evaluations of plaque, gingival crevicular fluid analysis, and the assessment of inflammatory mediator levels.

This model offers the advantage of noninvasively monitoring disease development in real time, enabling the study of the transition from a healthy to a dysbiotic state in human tissue (Bamashmous et al., 2021).

The classic full-mouth recording for human experimental gingivitis studies has undergone several modifications. These modifications include the extension of the induction phase from the traditional 3 weeks to 4 weeks. This extension aims to ensure the complete manifestation of gingivitis in most subjects, employing a half-mouth biofilm overgrowth model as detailed by Yates et al., (Yates, Shearer, Morgan, & Addy, 2003). Additionally, modifications have included adopting partial-mouth protocols and incorporating intra-oral acrylic stents into the experimental design. Studies by Putt et al. and Jones et al., showed that the partial-mouth experimental gingivitis model allowed unhindered development of plaque and gingivitis comparable to whole-mouth studies in which oral hygiene was suspended for 3 weeks. These findings indicate that such models can produce results comparable to those obtained from whole-mouth studies, showcasing the versatility and effectiveness of different experimental designs (Jones, Saxton, & Ritchie, 1990; Putt, Van der Weijden, Kleber, & Saxton, 1993).

Studies by Offenbacher et al. and Morelli et al. included the use of intraoral stents that covered selected teeth, allowing for the study of localized changes in biofilm overgrowth and inflammation.

These stents, worn during routine oral hygiene activities, served as a protective measure for specific teeth, allowing researchers to focus on localized changes in biofilm overgrowth and inflammation. (Morelli et al., 2014; Offenbacher et al., 2009).

Use of these stents has provided crucial insights into the localized accumulation of biofilm and its associated inflammatory response, thereby enhancing our understanding of the pathogenesis and resolution of gingivitis. Through these modifications, researchers have developed more refined methods to induce localized biofilm overgrowth and examine the ensuing inflammatory response, contributing significantly to our comprehension of gingival disease mechanisms.

1.4.3 Key Findings from the Human Experimental Gingivitis Studies

Research spanning several decades has highlighted the intricate relationship between plaque accumulation, gingival health, and the progression of experimental gingivitis. A study by Hillam and Hull (1977) demonstrated that both gingival and plaque indices show a positive correlation with plaque growth during the induction of experimental gingivitis, underscoring the direct link between plaque accumulation and gingival inflammation(Hillam & Hull, 1977). Further supporting this, Brecx et al.,(1987), validated the Plaque Index and Gingival Index as reliable markers for developing gingivitis, emphasizing their effectiveness in monitoring disease progression (Brecx, Schlegel, Gehr, & Lang, 1987). Additionally, a subsequent study by the same team in 1988 revealed that gingivitis developed more swiftly and intensely in older individuals. However, the gingival inflammation reverted to that of health upon resuming oral hygiene (Brecx, Lehmann, Siegwart, Gehr, & Lang, 1988). Expanding on these findings, Theilade (Theilade, 1996) offered a microbiological lens, using culture-dependent methods to explore gingivitis, while others like Bonfil et al. (1985) and Klinge et al. (1983) delved into its histopathological aspects and the process of recovery (Bonfil, Fourel, & Falabregues, 1985; Klinge, Matsson, & Attström, 1983).

The experimental gingivitis model has been employed to examine shifts in microbiological, immunological, and biochemical markers, shedding light on the dynamics of gingivitis onset and progression.

Early work by Moore et al., (1982) pinpointed *Actinomyces, Fusobacterium*, and *Streptococci* as the key etiological agents of gingivitis, with Syed (1978) documenting a microbial shift from *Streptococci* to *Actinomyces*-dominated plaque as gingivitis developed (Moore et al., 1982; Syed & Loesche, 1978).

Holm-Pedersen et al., (1975) found that gingivitis's onset and severity were more pronounced in older individuals, regardless of plaque microorganism consistency (Holm-Pedersen, Agerbaek, & Theilade, 1975). Dahlén (1992) proved that effective supragingival plaque control could modify the subgingival microbiota, suggesting a potential strategy for managing gingivitis.

In more recent studies, Kistler et al. observed that gingivitis is marked by changes in the bacterial community structure and an increased diversity of plaque microbiota(Kistler, Booth, Bradshaw, & Wade, 2013). While Al-Kamel (2019) identified specific bacterial species such as *TM7, Tannerella*, and *Cardiobacterium*, as linked to gingivitis development, indicating the role of microbes in disease progression. (Al-Kamel et al., 2019). A systematic review by Zemouri et al. (2019) also showed a significant microbiological shift in both supragingival and subgingival dental plaque towards a more pathogenic composition during gingivitis induction, with notable changes in the abundance of red and purple species, especially among smokers. This composition, however, tends to revert to a more normal state during the disease's resolution phase, highlighting the dynamic nature of the subgingival plaque microbiome throughout the course of experimental gingivitis (Zemouri et al., 2019). Genus-level changes to the microbiome were seen, where *Streptococcus, Neisseria, and Actinomyces* species were decreased in their abundance, and *Prevotella*, *Fusobacteria*, and *Porphyromonas* were significantly deceased in their abundance(Hall et al., 2023). These studies collectively emphasize the complexity and fluid dynamics of the subgingival plaque microbiome in the context of experimental gingivitis.

Overall, the body of research conducted thus far has significantly illuminated the complexity and dynamic nature of the subgingival plaque microbiome during experimental gingivitis. However, despite these advancements, there remains a substantial gap in our comprehension of the polymicrobial intricacies of gingivitis, particularly concerning how these microbial communities interact with the host at a cellular level during the internalization process.

To bridge this knowledge gap, there is a pressing need for human experimental gingivitis studies, especially those examining microbial internalization into the tissues; understanding the dynamics of host internalization and the changes in subgingival microbiome. These could reveal novel insights into disease progression and resolution mechanisms which in turn, could lead to the identification of new therapeutic targets aimed at modulating the host's response to microbial invasion or altering the microbial composition to prevent disease onset or hasten its resolution. Furthermore, such studies would provide invaluable information on the roles of individual bacterial

species and their consortia in the disease process. Such knowledge can pave the way for personalized dental care strategies to effectively manage or prevent gingivitis based on an individual's unique microbiome and host response profile.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1 Study Rationale

Gingivitis is a treatable form of periodontal disease caused by the overgrowth of oral microbes. Left untreated, gingivitis can progress to an irreversible disease known as periodontitis. The utilization of experimental gingivitis models, recognized for their reliability and effectiveness, allows for the real-time observation of periodontal disease development and progression.

Despite the widespread use of such models, a significant gap exists in our understanding of the microbial invasion of gingival tissues associated with plaque. Current research provides insights into the identities of bacteria that penetrate and become internalized within epithelial cells and the underlying connective tissue during the onset of gingivitis.

Although studies have investigated tissue invasion by known periodontal pathogens like *P. gingivalis, T. forsythia*, and *F. nucleatum*, (Abusleme, Hoare, Hong, & Diaz, 2021; Woelber et al., 2019) a comprehensive analysis of the internalized microbiome throughout the stages of experimental gingivitis remains largely unexplored. To date, no study has thoroughly characterized the subgingival plaque microbiome in tandem with the microbiome that invades and resides within host tissues during gingivitis's development and resolution.

Our research aims to fill this crucial gap by examining the changes in the sub-gingival microbiome and the internalization of specific bacterial subsets into gingival tissues. We intend to explore both the spatial (comparing subgingival plaque to gingival tissue compartments) and temporal dynamics of bacterial communities (changes in both compartments over the development and resolution of gingivitis).

By conducting a "paired" analysis of these compartments, our study seeks to uncover pivotal insights into the microbial community shifts contributing to the invasion during gingivitis. This approach promises to deepen our understanding of the complex interactions between the subgingival microbiome and host tissue internalization, offering potential pathways for targeted interventions in managing and preventing periodontal diseases.

2.2 Hypothesis

We hypothesize that during experimentally induced gingivitis, a subset of subgingival plaque bacteria internalizes into host gingival epithelial and connective tissues.

2.3 Objectives

2.3.1 To characterize and compare the compositional and functional changes in the subgingival plaque microbiome and the internalized microbiome within gingival tissue compartments during the development and resolution of experimental gingivitis.

2.3.2 To examine key bacterial species and identify interbacterial interactions that facilitate significant microbial community shifts and the invasion of gingival tissues during the induction of experimental gingivitis.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Design and Ethics Approval

The study protocol received ethical approval from the Health Research Ethics Board-Health Panel at the University of Alberta (Approval Number: PRO00112019). Detailed information regarding the study's objectives, procedures, potential risks, discomforts, and benefits was thoroughly communicated to participants verbally and in writing. Before undergoing dental examination and sampling, written informed consent was secured from each participant, ensuring their voluntary and informed participation in the study.

Participant Demographics: The study analyzed samples from 22 patients who successfully completed the research. These participants were recruited from the School of Dentistry at the University of Alberta, with their ages ranging between 34 and 60 years. The demographics of the study population are summarized in **Table 1**. Consultations and treatments for all involved patients were conducted at the Graduate Periodontics Clinic within the Kaye Edmonton Clinic, located at the School of Dentistry, University of Alberta.

The following were the inclusion criteria for a patient to participate:

(i) At least 18 years of age and good general and oral health [No presence or history of diabetes or cardiovascular diseases].
(ii) At least 20 natural teeth present
(iii) Baseline mean gingival index of less than or equal to 1 (according to the scoring by the modified gingival index [MGI]).
(iv) No history of Periodontitis
(v) Not undergoing orthodontic treatment

Conditions that would exclude an individual from participating were:

(i) History of conditions requiring prophylactic antibiotic coverage before this study
(ii) Use of antibiotic, anti-inflammatory, or anticoagulant medication within 1 month before the study
(iii) History of smoking or tobacco use

(iv) Participation in another oral study involving oral care products concurrently or within 30 days of beginning this study.

(v) Pregnant or lactating

(vi) Significant oral tissue pathology (excluding gingivitis).

(vii) Moderate to advanced chronic periodontitis or other form of periodontal disease; and

(viii) An underlying genetic or immunologic condition that might influence the study (e.g., diabetes or immunodeficiency).

Table 1: Patient Demographics

Demographic Variable	N	Mean (SD) / Frequency (%)
Total Participants	22	
Age (years)		39.14 (7.18)
Gender		
- Female		12 (54.5%)
- Males		10 (45.5%)
Education Level		
- College Graduate		22 (100%)
- High School		--
Health Status		
- Systemically Healthy		22 (100%)
- Periodontally Healthy		22 (100%)

3.2: Study Design

Initial Visit and Procedures: A simplified illustration of the steps involved in the study is outlined in **Figure 3**. Patients meeting the inclusion criteria underwent a comprehensive periodontal examination conducted by a periodontology resident (AYB) during their first visit including recording probing pocket depths using UNC 15 periodontal probe [PPDs] (in millimeters), Bleeding on probing [BOP](Ainamo, 1992) (yes or no) at a total of six sites per tooth on both the buccal and lingual marginal gingiva. Recording indices such as Modified Quigley Hein Plaque Index [MQHPI](Quigley & Hein, 1962; Turesky, 1970) and Papillary Marginal, Attachment [PMA] Index (Schour & Massler, 1947). Subgingival plaque and gingival crevicular fluid (GCF) samples were collected from the interproximal areas of the first molars. If the first molar was missing, samples were taken from the second molar. This session also included recording

impressions of the upper and lower jaws, followed by performing scaling and root planing, and instructions for proper oral hygiene were provided.

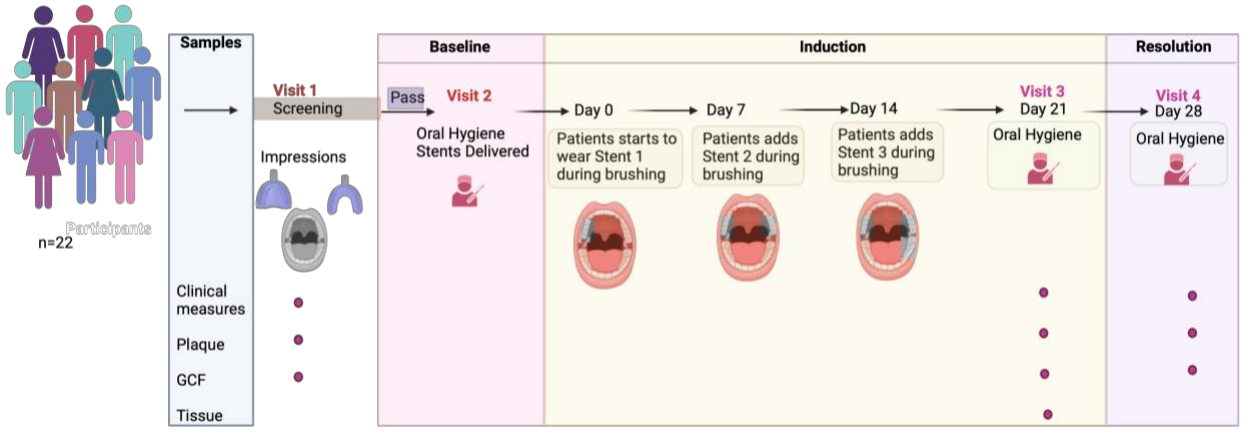

Figure 3: Study Design for Experimental Gingivitis Induction and Resolution. This figure illustrates the study design used to assess the induction and resolution of experimental gingivitis in the participants. The study comprised four visits: Visit 1 involved screening participants to ensure they were systemically and periodontally healthy. Visit 2, at baseline (Day 0), included oral prophylaxis, and the delivery of stents. Participants began wearing Stent 1 during brushing. During the induction phase, at Day 7 and Day 14, participants added Stent 2 and Stent 3, respectively, during brushing. Visit 3, on Day 21, involved sample collection, a clinical exam, and administration of professional oral hygiene (OH). Finally, during the resolution phase (Visit 4, Day 28), a clinical exam and further oral hygiene were performed where applicable. The design ensures systematic evaluation of clinical parameters and microbial changes at each stage. The figure was created using Biorender.com.

Acrylic Stent Fabrication: The lab at the Kay Edmonton Clinic created the acrylic stents to shield the posterior teeth—from premolars to the distal-most molar across three of the four quadrants, extending 2mm beyond the gingival margin. Patients chose the control quadrant to maintain oral hygiene during the induction phase based on personal preference. The stents were designed to block the cleaning of the specified quadrants during the study and were worn exclusively during daily oral hygiene routines.

Subsequent Visits and Sample Collection: Within a week of the initial visit, participants returned for a second visit to start the study officially. The lab fabricated stents were delivered. Each participant received the same toothbrush and toothpaste (soft-bristled Colgate toothbrush and Crest Gum Detoxify toothpaste) and was instructed to avoid using any other oral care products (such as interdental cleaning aids, mouthwash, or chewing gum) during the study in the areas involved in the study. The placement of stents in the participants' quadrants was determined random (an online randomizer tool – randomizer.org), with the control quadrant chosen by the patient. The participants were given a written schedule for wearing the stents to the assigned quadrant. Starting from day 0, one stent was worn during brushing and flossing. On day 7, a second stent was introduced, and by day 14, a third stent was added. By the end of the study, the first quadrant (day 0 start) would have had 21 days without oral hygiene, the second (day 7 start) 14 days, the third (day 14 start) 7 days, and the quadrant without a stent served as the control. The participants were scheduled for their third visit on day 21. Subgingival plaque, sulcular tissue, and GCF samples were collected for all quadrants. Following the sample collection, all patients underwent scaling and root planing. A week later (visit 4), subgingival plaque and GCF samples were collected, followed by another round of scaling and root planing. This would be followed by subsequent visits a week apart until their MGI score was at 0.

3.3: Subgingival plaque sampling

Subgingival plaque samples were collected from the same sites during the study by inserting one sterile endodontic paper point (Dentsply Caulk, Milford, DE) into the sulcus of each tooth for 15 seconds. Sulcular tissue samples were collected similarly from the allocated quadrant of each participant before, during and after the onset of the experimental gingivitis study under local anesthesia. The subgingival plaque samples at each time point were pooled in a microcentrifuge

tube containing RNA*Later* (Invitrogen) and stored at -20°C until further sample processing. Details are described in the next section.

3.4: Gingival tissue sampling and removal of attached bacteria from tissue samples

The tissue samples were excised from the mesial and distal interproximal areas of the first or the second molars, using the previously published ENAP procedure [**Figure 4 A and B**] (Yukna, 1978).

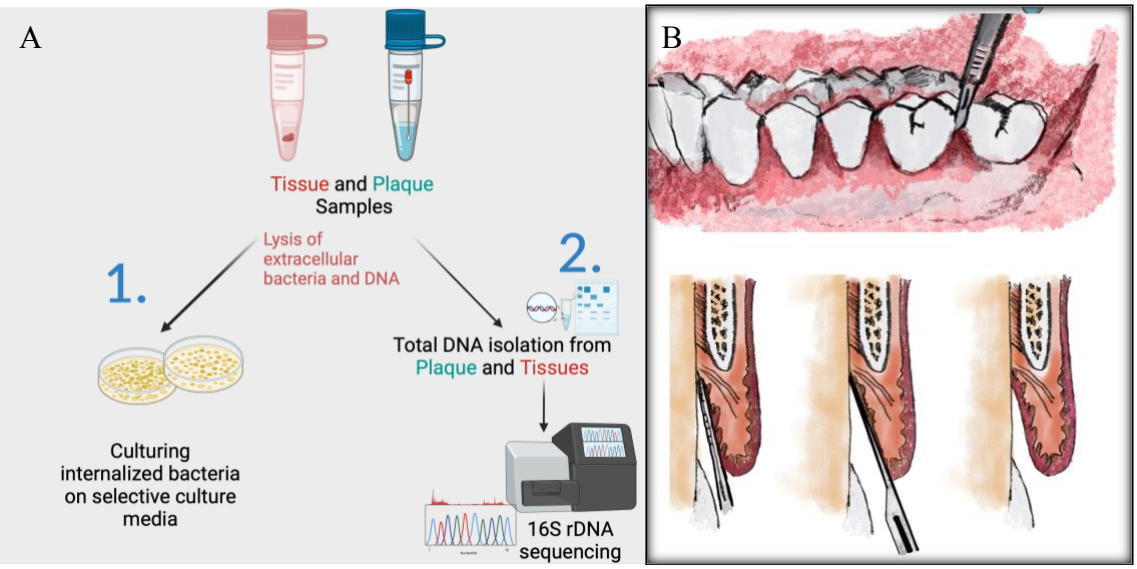

Figure 4: A. Sample Analysis Strategy and B. Technique for Obtaining Tissue Samples Using Excision New Attachment Procedure (ENAP). This figure illustrates the technique for obtaining tissue samples using the Excision New Attachment Procedure (ENAP), a method adapted from the original surgical design by Yukna et al., 1978. The top panel shows the initial incision along the gingival margin to access the periodontal pocket. The bottom sequence illustrates the subsequent steps: excision of the pocket epithelium, removal of granulation tissue, and the final stage where the pocket epithelium is completely removed to allow for new attachment. This procedure facilitates the collection of gingival tissue samples for microbiome analysis. The figures were drawn using the Biorender.com and Procreate.com.

The tissue samples were obtained only for the induction period of the study [Control, Day 0, Day 7, Day 14, and Day 21]. The samples, except for three patients, were subdivided into two parts: One was used for DNA isolation, and the other was used for RNA isolation. For three patients, a third fraction was used for culturing to examine bacteria internalized into gingival tissues using commercially available culture media. Except for the sub-samples intended for RNA isolation, all the samples were subjected to the following pre-processing: The adherent extracellular bacteria were removed by washing thrice with PBS and treatment with 100 µg/ml gentamicin for 1 h (Lai & Walters, 2013). This was followed by washing thrice with 1X PBS. Next, to remove any contaminating extracellular bacterial DNA on the tissue surface, samples were treated with DNase I. This was followed by washing thrice again with 1X PBS. The samples were then stored in RNA*Later* until further use.

For tissue samples intended for RNA isolation, the samples were washed thrice with 1X PBS followed by storage in RNALater and snap freezing until at -80ºC until further use. The tissue samples were then processed for DNA and RNA extraction using Qiagen's All Prep DNA/RNA kit [QIAGEN, Hilden, Germany] as per the manufacturer's recommendations. The Isolated DNA was quantified and subsequently subjected to 16S rDNA sequencing to identify internalized bacteria. Isolated total RNA was stored in -80 ºC for downstream processing.

3.5: Materials used in the study

3.5.1 Reagents

The following reagents were used in the study: Gentamicin (50 mg/ml) purchased from Sigma Aldrich (Cat #G1397); Qiagen AllPrep Bacterial DNA/RNA/Protein Kit (Cat# 47054; QIAGEN, Hilden, Germany); Qiagen RNeasy Mini Kit (Cat. #74104); Lysozyme, purchased from Fisher Scientific (Cat #190082); DNAse I, Purchased from Thermo Scientific (Cat # PI90083). DNA or RNA was quantitated using the Qubit (Thermo Fisher Scientific) with the 1X dsDNA HS Assay Kit (Cat #Q33230, Thermo Fisher Scientific) or the RNA HS Assay Kit (Cat #Q32852, Thermo Fisher Scientific), respectively.

3.5.2 Media for bacterial culture

The following selective media were purchased commercially from Anaerobe systems: *Porphyromonas gingivalis* Agar (selective for *P. gingivalis*; Cat. #AS-6422); Tryptic Soy Agar with n-Acetylmuramic acid – TSA-NAM (selective for *T. forsythia*; Cat. #AS-6421); Fusobacterium Selective Agar – FSA (selective for *F. nucleatum*; Cat. #AS-6427); Tryptic Soy-Serum-Bacitracin-Vancomycin Agar – TSBV (selective for *A. actinomycetemcomitans*; Cat. #AS-648). The plates were stored at room temperature until use and acclimatized to anaerobic conditions overnight before use. They were then stored at 4°C after exposure to ambient air until use.

3.5.3 Buffers

PBS: 137 mM NaCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 2.7 mM KCl (pH 7.6)

3.5.4 Tissue homogenization and bacterial culture

Bacteria internalized into gingival tissues were cultured using previously published protocols with minor modifications (Baek et al., 2018; Medapati et al., 2021; Schmit, Patton, & Gilmore, 2011). Briefly, gingival tissue samples were washed three times with 1 mL PBS to remove non-adherent bacteria. Tissues were then incubated with gentamicin (100µg/ml) at 37 °C for 1 hour to eliminate any remaining surface-attached bacteria. The gentamicin wash was plated as a negative control to confirm the efficacy of surface bacteria removal.

Next, to remove any contaminating extracellular bacterial DNA on the tissue surface, samples were treated with DNase I. Tissues were then washed twice more with 1 mL PBS before homogenization by mechanical douncing in 0.025% saponin solution. The homogenized tissue lysates were utilized for both bacterial culture and DNA extraction to evaluate the intracellular microbial composition. This optimized protocol allows the characterization of the microbiome internalized within gingival epithelial and connective tissue cells, excluding extracellular surface bacteria.

3.5.5 Sample size

This trial was a pilot study due to the absence of prior data. Sample size calculations were derived from previous studies investigating clinical and microbiological assessments in human experimental gingivitis. Participants acted as their own control, with a hypothesized medium effect size (Cohen's d = 0.5). Given the nature of this study utilizing 16s rDNA sequencing to analyze

bacterial shifts during experimental gingivitis, moderate to large effect sizes were anticipated (Cohen effect size d=0.5 for moderate, 0.8 for large, therefore d=0.65 was used). This consideration aimed to balance the ability to detect statistical significance while detecting clinically and microbiologically meaningful differences. Hence, employing a significance level (alpha) of 0.05 using a paired t-test and assuming a medium to large effect size, a sample size of 22 participants was determined to achieve an 80% power to detect a statistically significant difference.

3.5.6. 16srDNA Sequencing

Amplicon sequencing was performed at the Genome Quebec core facility by amplifying the V1-3 using the 27F/519R primer, with the following sequences:

Forward primer 5'-
ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAKRGTTYGATYNTGGCTCAG- '3,
and
Reverse primer 5'-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGTNTBACCGCDGCTGCTG- '3.

Additionally, the V4-5 region was also sequenced using the 515bF-926R primer with the following sequences:

Forward primer 5'-
ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTAA- '3
and
Reverse primer 5'-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGYCAATTYMTTTRAGTTT- '3.

Sample pre-processing was performed using an open-source, fully automated pipeline developed by the lab (FAVABEAN: available at github.com/khalidtab/favabean/) on a computationally capable server (20 cores, 128 gigabytes memory) Mac Studio. Briefly, amplicon sequence variants were generated from the sequences by determining the best parameters for merging with Figaro (Weinstein, Prem, Jin, Tang, & Bhasin, 2019). Next, sequences were denoised, and condensed with DADA2 (Callahan et al., 2016). Taxonomic identification was performed against the Human Oral Microbiome Database (HOMD) (Chen et al., 2010). Sequences that matched a species in HOMD at 100% similarity were designated as a species.

3.5.7 Statistical analysis

Each participant was treated as a statistical unit, and all statistical tests were used with alpha=0.05. All analyses were conducted using either R, or GraphPad Prism V.10 software packages. Clinical parameters such as PPDs, MQHPI, and PMA Index were assessed using repeated measures ANOVA or the Friedman test if the data distribution was not Gaussian. Beta diversity was measured through Isometric Log-Ratio (PhILR) transformation (Silverman, Washburne, Mukherjee, & David, 2017). This analysis was calculated using an open-source fully automated pipeline developed by the lab (FALAPhyl: available at github.com/khalidtab/falaphyl/). Typical beta diversity measures cannot account for the repeated measures of sites. To reduce this confounder into a single time point, we performed a dimensionality reduction by constructing a tensor that bisects these timepoints into a single distance (Shi et al., 2024). As such, each "sample" represents the trajectory of microbial change in a site. Alpha diversity metric was measured as a phylogeny-aware diversity during assembly (designated as *D*) (Darcy et al., 2020). Linear mixed model analyses were performed using the R package "lmer". Network analysis was done using the package eLSA (extended local similarity analysis) after transformation of the data using a robust centroid log ratio, then graphed using Gephi (Bastian; Martino et al., 2019; Li C. Xia et al., 2011). Only edges that are statistically significant ($p<0.05$) were retained. Differential abundance was through the package Tweedieverse (Mallick et al., 2022) using the Zero-inflated Compound Poisson (ZICP) distribution per package developer recommendation for microbial compositional data
(https://github.com/himelmallick/TweedieLabs/blob/main/R/Tweedieverse_microbiome.html).

CHAPTER 4: RESULTS

4.1: Proof of Concept: Bacteria internalization in the gingival tissues occurs during experimental gingivitis

Culture-dependent methods help isolate viable bacterial species. In our study, we aimed to investigate whether the presence of periodontal pathogens in the gingival tissues is due to invasion into the periodontal pathogens, or the uptake of microbiome is due to passive presence in immune cells as a part of immune system antigen processing. This proof-of-concept method also serves to demonstrate whether these pathogens are able to survive the transport and processing with gentamicin, as this antibiotic is bactericidal, which requires viable bacteria for it to be effective. Destruction of extracellular bacteria leaves inter-tissue and intracellular bacteria.

Excised tissues from three patients were utilized in this proof-of-concept. We used selective media designed to culture previously identified internalizing species. Although standardized buffers and protocols were used, variations in tissue weights prevented standardization. Therefore, these culture tests only serve as confirmation of growth and are not to be compared to each other. The results of the culture confirmed internalization into tissues during induction of inflammation. Control gentamycin washings confirmed the absence of extracellular bacteria and therefore the successful of eradication of extracellular bacteria. Therefore, based on these results, further analyses of the samples were done using culture-independent methods (metataxonomics) to further identify the microbiome of gingival tissues.

Figure 5 demonstrates that all the four-culture media supported bacterial growth at various stages of the gingivitis study [Days 7, 14, 21, and control], with no growth in the negative controls. However, it was not possible to quantify colony-forming units (CFUs) due to the inconsistent sample sizes of the homogenized tissues.

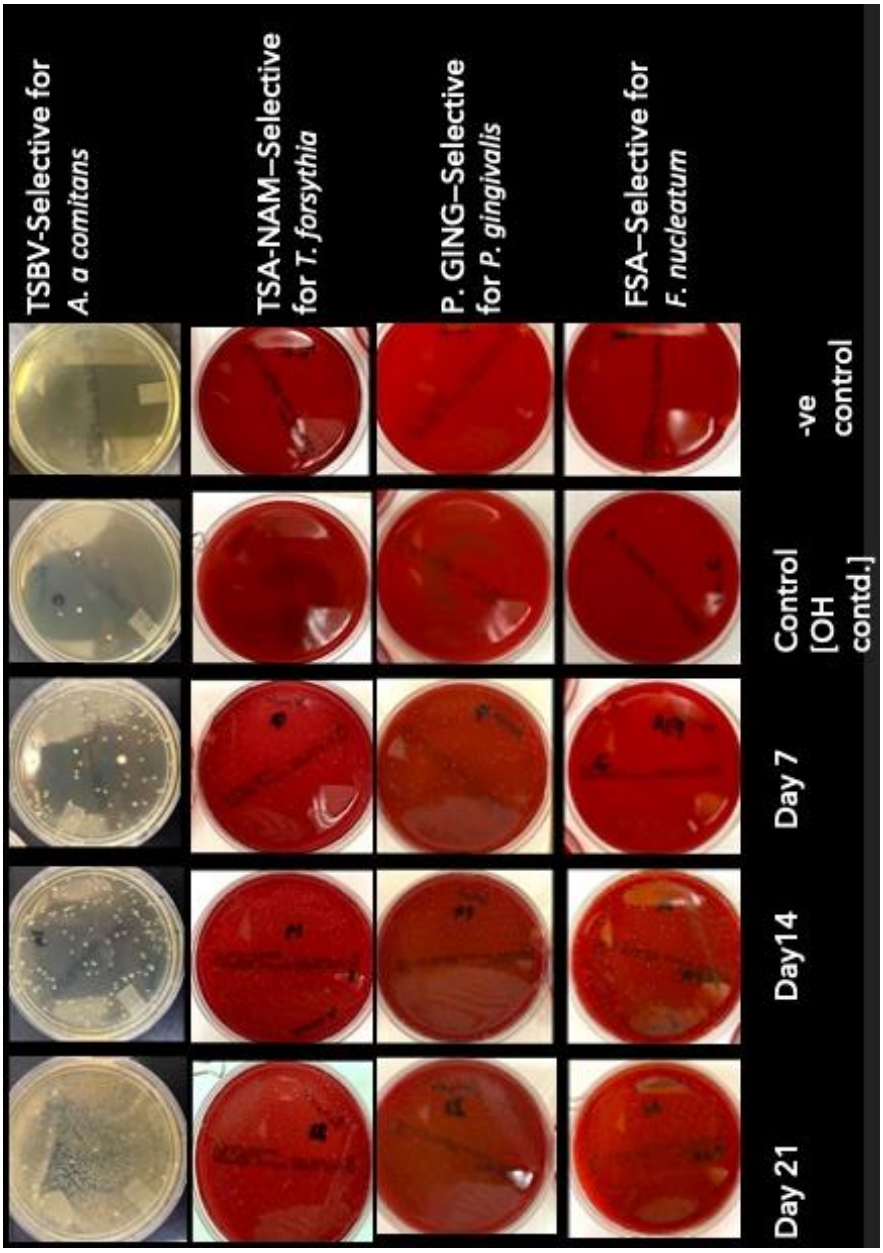

Figure 5: Culture-dependent identification of bacteria internalized into gingival tissues. Tissue samples at various time points in the study [Day 7, 14, 21 and Control] were first treated with gentamicin to remove any externally attached bacteria. This was followed by homogenization and plating on culture plates selective for *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Tanerella forsythia*. The PBS wash obtained from the tissues after treatment with gentamicin were plated as negative controls.

4.2: Assessment of changes in clinical parameters during induction and resolution of gingivitis.

4.2.1. Pre-operative assessment of clinical parameters (effectiveness of randomization)

To ensure there were no systematic differences between the different quadrants assigned for each time point through randomization, especially considering the involvement of both the maxilla and mandible, we evaluated several clinical parameters. These included probing pocket depth (PPD), modified Quigley-Hein plaque index (MQHPI), papillary marginal attachment index (PMA Index), and bleeding on probing (BOP). This was crucial as patient preferences for certain areas could potentially influence clinical presentation.

PPD was analyzed using linear mixed models with the following formula:

- ppd = lmer(as.numeric(ppd) ~ quadrant + (1|patientID) + (1|surface)

This formula assesses pre-operative PPD by comparing inter-quadrant measurements for the teeth included in the study. PPD served as the dependent variable, quadrant as the independent variable (fixed effect), and patient ID and surface level as random effects variables. Utilizing this linear mixed model, the following scaled residuals were obtained. The t-tests used Satterthwaite's method:

REML criterion at convergence: -1785.8

Table 2:Scaled Residuals

Min	1Q	Median	3Q	Max
-6.442e-06	0.000e+00	0.000e+00	7.244e-07	7.244e-07

Table 3:Random effects

Number of observations: 88
Groups: PatientID (22); surface (4)

Group	Variance	Std. Dev.
PatientID	1.071e-02	1.035e-01
Surface	0.000e+00	0.000e+00
Residual	9.433e-14	3.071e-07

Table 4:fixed effects

Effect	Estimate	df	t value	Pr (>|t|)
Intercept	2.89879	0.03273-0.21180	88.573	0.288
QuadrantQ2	0.10113	0.05669-0.21180	1.784	0.656
QuadrantQ3	0.10108	0.06813-0.21180	1.484	0.681
QuadrantQ4	0.10111	0.06123-0.21180	1.651	0.666

Table 5:Correlation of Fixed Effects

	Intercept	QuadrantQ2	QuadrantQ3
QuadrantQ2	-0.577		
QuadrantQ3	-0.480	0.277	
QuadrantQ4	-0.534	0.309	0.257

As shown, using quadrant 1 as the reference, there was no statistically significant difference in PPD between the quadrants pre-operatively. Additionally, no significant differences were found in PPD or BOP at the pre-operative sites ($p > 0.05$). However, plaque levels were significantly higher in the maxilla compared to the mandible pre-operatively. Given that prophylaxis was performed on all individuals prior to the study's commencement, this finding is likely incidental and of no clinical significance (p-value = 1.286e-06, Fisher's exact test). Moreover, the amount of accumulated plaque may be visible but minimal to the point that they do not have any influence on the BOP.

4.2.2: Clinical Parameters Evaluation During Experimental Gingivitis Induction/Resolution

Upon cessation of oral hygiene, all test sites within the study exhibited expected outcomes — plaque accumulation was observed, and quantified using the MQHPI, other measures including PPD, BOP and PMA indices were recorded as well (**Figures 6 and 7**). Plaque accumulation, as measured by MQHPI, increased during the induction phase of gingivitis, corroborating the clinical signs of gingival inflammation observed. Plaque levels at day 21 was statistically significantly greater than those in control groups ($p<0.001$). At baseline and the resolution phase, MQHPI scores were significantly lower indicating the reversal of experimental gingivitis ($p<0.05$ and $p<0.05$). The probing depths remained relatively stable across the various time points, with a significant increase observed on Day 21 ($p<0.05$) of the induction phase and no change in the baseline or the

resolution phases of gingivitis. This suggests that the induced gingivitis did not result in substantial changes in probing depths. The BOP increased during the experimental gingivitis phase, for both Day 14 and Day 21, ($p<0.01$ and $p<0.001$, respectively) reflecting the inflammatory response to plaque accumulation. However, BOP levels in the resolution phase were non-significantly changed from the control, indicating a successful return to periodontal health post-intervention. This dynamic change highlights the role of BOP as an indicator of gingival inflammation and its utility in monitoring periodontal health.

During the induction phase of gingivitis, there was a noticeable increase in the papillary and marginal gingival inflammation, indices suggestive of gingival swelling and inflammation. This change was statistically significant for all time points compared to the pre- and post-operative measurements ($p<0.01$). Upon resolution, the papilla and margin values returned to levels comparable to the baseline measurements. Similarly, during the inflammatory phase, the attachment index increased. Alterations in the attached gingiva became apparent on day 21 for certain participants ($p<0.05$). However, following the resolution phase, the attachment index improved significantly, approaching the values observed before the induction of gingivitis.

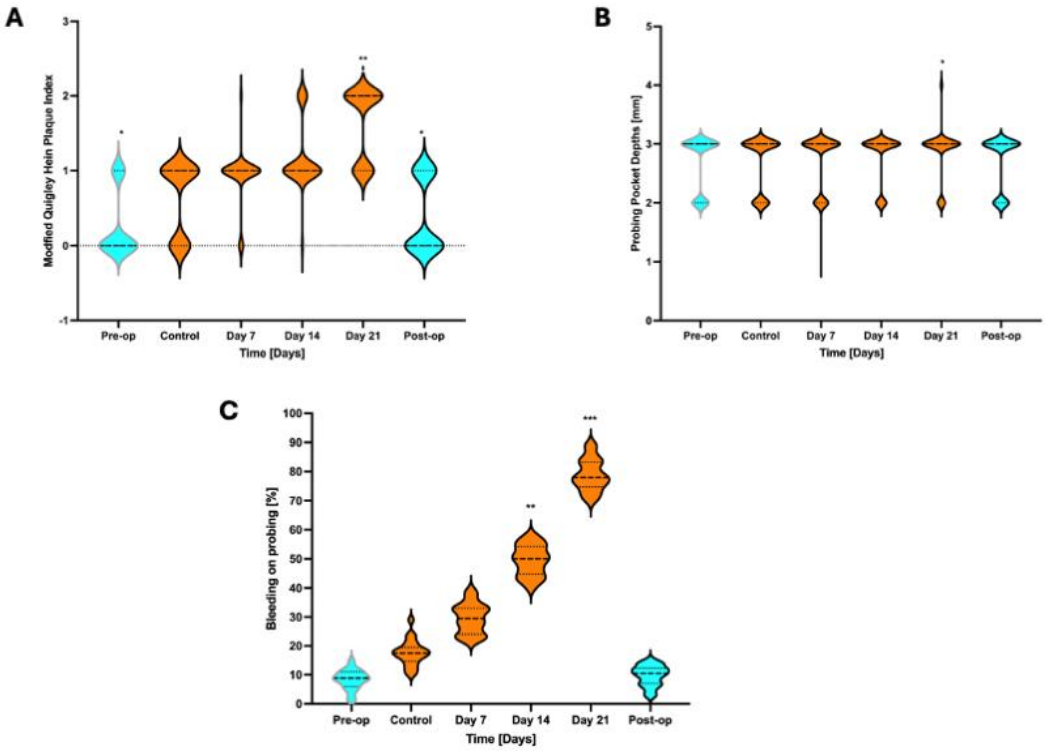

Figure 6: Dynamic changes in periodontal health over the induction and resolution of experimental gingivitis. The box and violin graph depicts the changes in various parameters over various time points in the study.A. Depicts dental plaque accumulation, assessed by the modified Quigley-Hein Plaque Index (MQHPI) and graph on right[B] shows the mean probing pocket depths (PPDs) as measured in milimeters, The bottom graph [C] presents data as percent change in bleeding on probing (BOP %). The induction phase is shown in yellow. The pre and post-operative measures [baseline and resolution phases] are shown in turquoise. A decrease in plaque accumulation, PPDs and BOP % indicates an improvement or a return toward normal gingival health following intervention. The statistical significance is indicated as "*"($p<0.05$) , "**" ($p<0.01$), and "***" ($p<0.001$). The graphs were analyzed using multiple comparisons following fitting a mixed effects model through repeated measures ANOVA, demonstrating the temporal changes and the effect of the intervention over the study period.

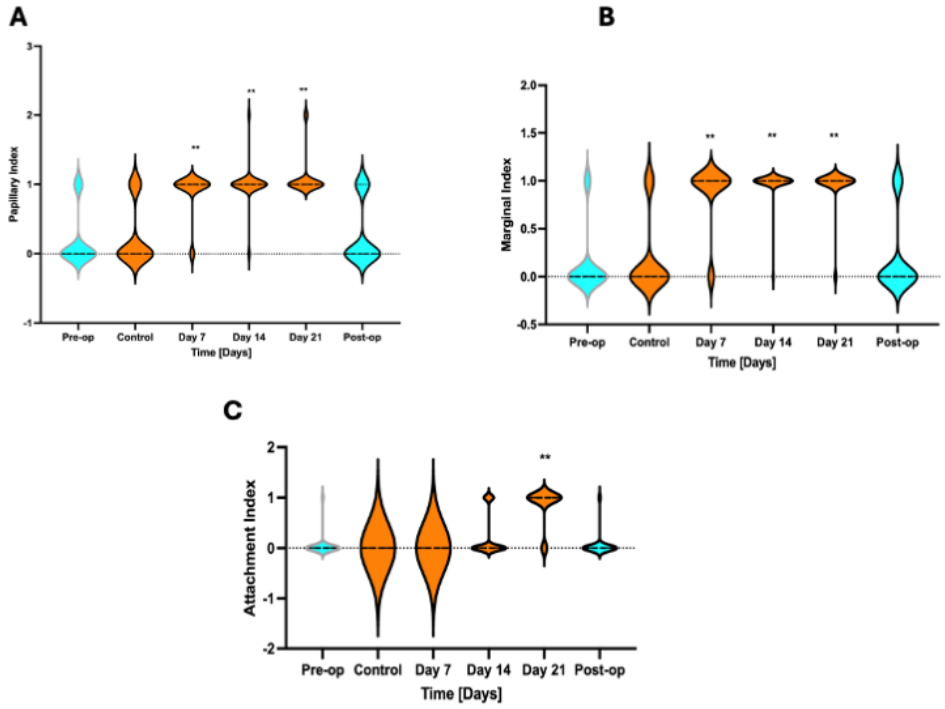

Figure 7: Papillary Marginal Attachment Index during Induction and resolution of experimental gingivitis. The attached box and violin plots provide a visual representation of the progression and resolution of experimental gingivitis as indicated by the papillary[**A**], marginal[**B**], and attachment[**C**] indices. The top panel displays the Papillary Index, where an increasing trend during the induction phase (Turquois) signifies the deterioration in papillary health which then declines during the resolution phase (Yellow). The middle graph represents the Marginal Index, following a similar trend of increase during induction and decrease during resolution, indicating a response to treatment. Finally, the bottom panel shows the Attachment Index, which increases significantly on Day 21, reflecting potential longer-term changes to the attachment because of the gingivitis induction, before showing signs of recovery. '**' denotes statistical significance levels($p<0.01$), and '*' indicates $p<0.05$.

4.3: Assessment of changes in microbial diversity and composition over the course of development and resolution of human experimental gingivitis

4.3.1 Alpha diversity analysis of plaque and gingival tissue microbiomes

The alpha diversity analysis of the plaque bacterial microbiome and those internalized into host gingival tissues was conducted to understand the impact of oral hygiene cessation and the resulting inflammation on microbial richness and evenness. Chao1 index (Chao, 1984) was used to estimate species richness, while the Inverse Simpson (Simpson, 1949) and Shannon's diversity indices (Shannon, 1948) were used to assess for both richness and evenness while accounting for abundances of the taxa. These analyses were performed at various time points, including pre-operative (pre-op), control, Day 7, Day 14, Day 21, and post-operative (post-op, 7 days after resuming oral hygiene) for plaque samples, and control, Day 7, Day 14, and Day 21 for tissue samples. For analysis and comparisons, the Friedman's non-parametric test for repeated measures was used.

The Chao1 analysis for plaque (**Figure 8. Panel A**) indicated no significant changes in species richness across the different time points during induction, suggesting stability in the plaque microbiome during the induction phase of experimental gingivitis. The Chao1 index for gingival tissues (**Figure 8. Panel B**) also showed no significant change in species richness from control to Day 21, indicating a selective environment within the tissues as gingivitis progressed. Similarly, the Inverse Simpson index (**Figure 8. Panels C and D**) and Shannon's diversity index (**Figure 8. Panels E and F**) revealed no significant changes in plaque samples over time, reflecting stable diversity and evenness in the plaque microbiome. Both indices also showed stability in observed ranks within gingival tissue samples, highlighting a stability in diversity and evenness as the inflammation intensified. These findings suggest that while the plaque microbiome changes during the study period, the gingival tissue microbiome remains stable, indicating that the tissue environment is selective to the survival of a few taxa.

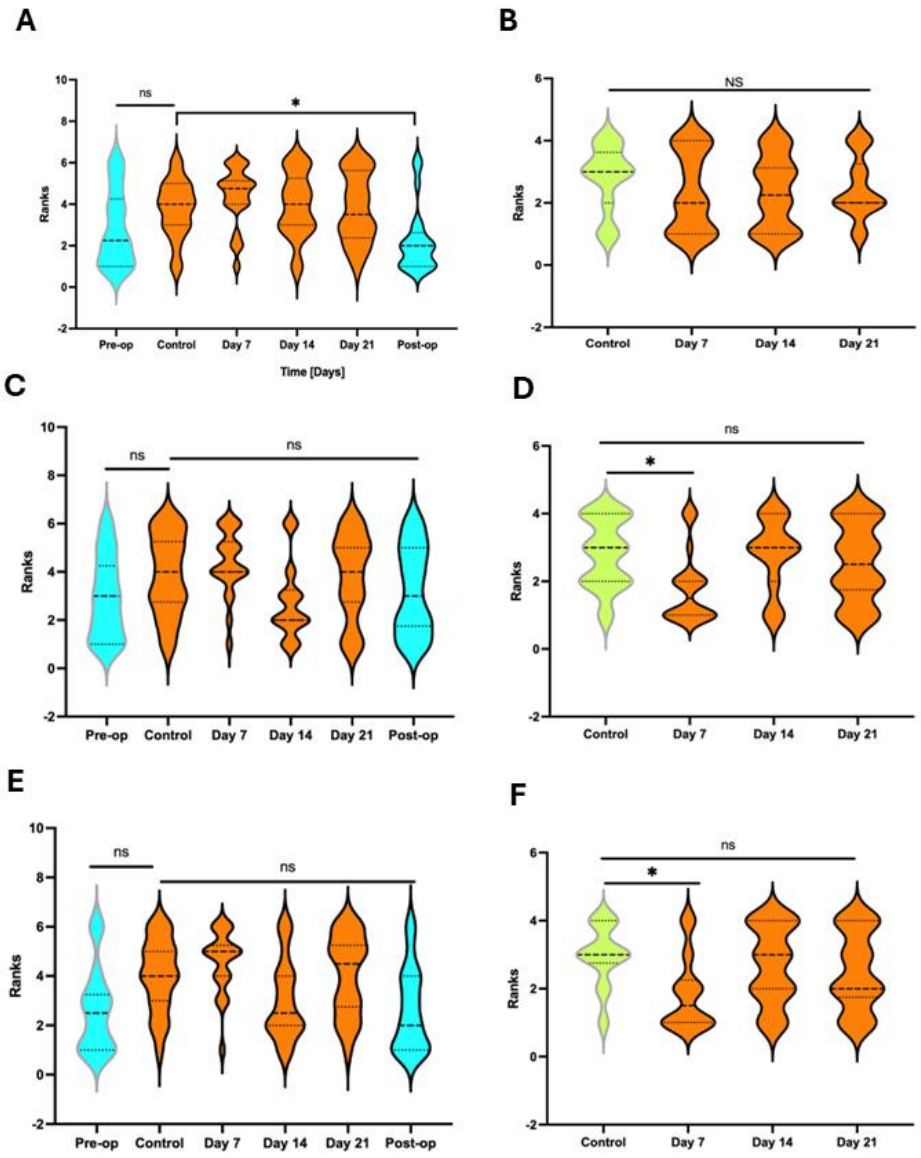

Figure 8: The alpha diversity of the plaque and tissue during experimental gingivitis. The figures shows plaque bacterial taxa [A, C and E] and those internalized into host gingival tissues [B,D and F] assessed using Chao1[Top], Inverse Simpson[Middle], and Shannon's diversity[Bottom] indices. These analyses were performed at various time points, including pre-operative (pre-op), control, Day 7, Day 14, Day 21, and post-operative (post-op, 7 days after resuming oral hygiene) for plaque samples, and control, Day 7, Day 14, and Day 21 for tissue samples.

The discovery that phylogenetic relatedness between taxa is an important contributor to the environmental niche occupation has greatly enhanced our understanding of the microbiome/environment relationship. This has been demonstrated with the popularity of the various Photogenically-aware dissimilarity measures (collectively known as UniFrac dissimilarity methods) used in microbial ecology (Lozupone, Lladser, Knights, Stombaugh, & Knight, 2010; McDonald et al., 2018; Wong, Wu, & Gloor, 2016). In non-phylogenetically aware methods, each taxon is considered to be wholly dissimilar from the other microbes in the microbiota. On the other hand, Unifrac dissimilarity methods account for the phylogenetic relatedness by rescaling these dissimilarities based on the phylogenetic tree distances between the bacteria. Regarding microbial colonization in human niches, Darcy et al constructed an algorithm, Phylogenetic Diversity during Assemblage (*D*), which showed that human microbiome generally follows nepotistic acquisition. That is, when a bacterium is present in a niche, it is more likely that a phylogenetically related bacterium would be able to colonize the area (termed, microbial nepotism). As such, we wanted to examine whether this microbial nepotism phenomenon is present in subgingival plaque and gingival tissues. We used the Phylogenetic Diversity during Assemblage (*D*) algorithm on the experimental gingivitis samples (days 7 through 21) **(Figure 9A)**. The results demonstrated that plaque's microbial recruitment follows a nepotistic microbial recruitment pattern ($D \neq 0$, $p = 0.005$). On the other hand, microbial recruitment in gingival tissues did not display the same pattern, where nepotistic recruitment was not statistically different from random assemblage ($p=0.053$, **Figure 9A**). The strength of the nepotistic effect of microbial assembly in plaque (estimate of $D = -0.33$) is statistically different from that of tissues (estimate of $D = -0.154$) ($p = 1.87e\text{-}22$). When the assembly effect is scaled to account for the relative abundance of the recruited species **(Figure 9B-C)**, both plaque and tissues displayed a significant nepotistic recruitment effect.

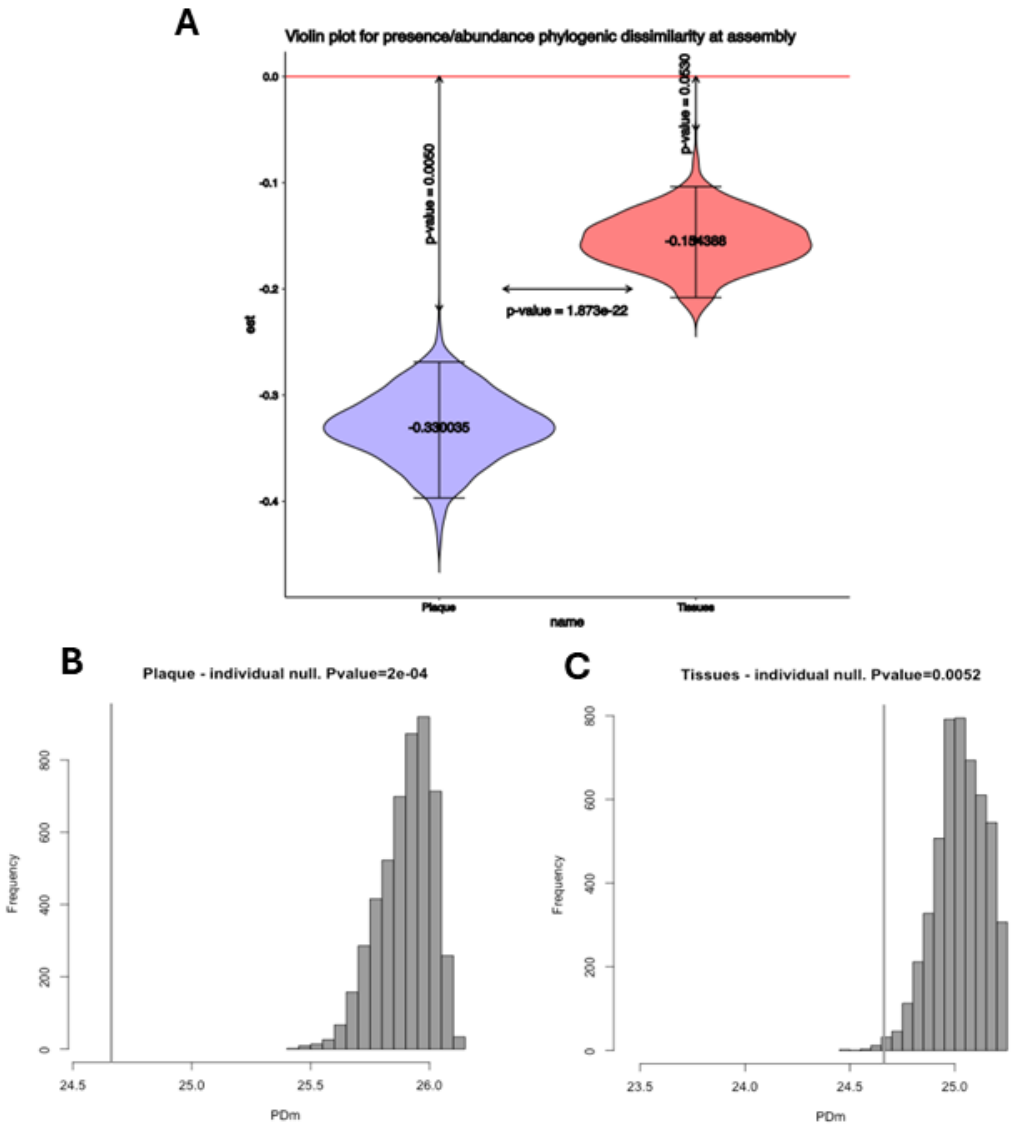

Figure 9: Phylogenic diversity (nepotism) during assemblage (*D*) as a presence/absence function. A) Violin plot of plaque (blue) and gingival tissue (red). Plaque displayed microbial nepotism, while gingival tissues did not display similar microbial maturation pattern (p=0.053). B) When accounting for relative abundance of recruited species, plaque displayed significant nepotism effect (p=2e-4), and C) the same nepotism effect was seen in tissues (p=0.0052)

4.3.2 Beta diversity analysis

We further investigated the microbial composition in the two niches. We first constructed a dissimilarity matrix by utilizing a tensor to account for the trajectory of microbial acquisition in the samples, so that each trajectory is represented by a single sample (a dot in **Figure 10**). The constructed dissimilarity demonstrated that the microbial trajectory in the plaque and gingival tissue niches are distinct from each other ($p<0.05$, ADONIS).

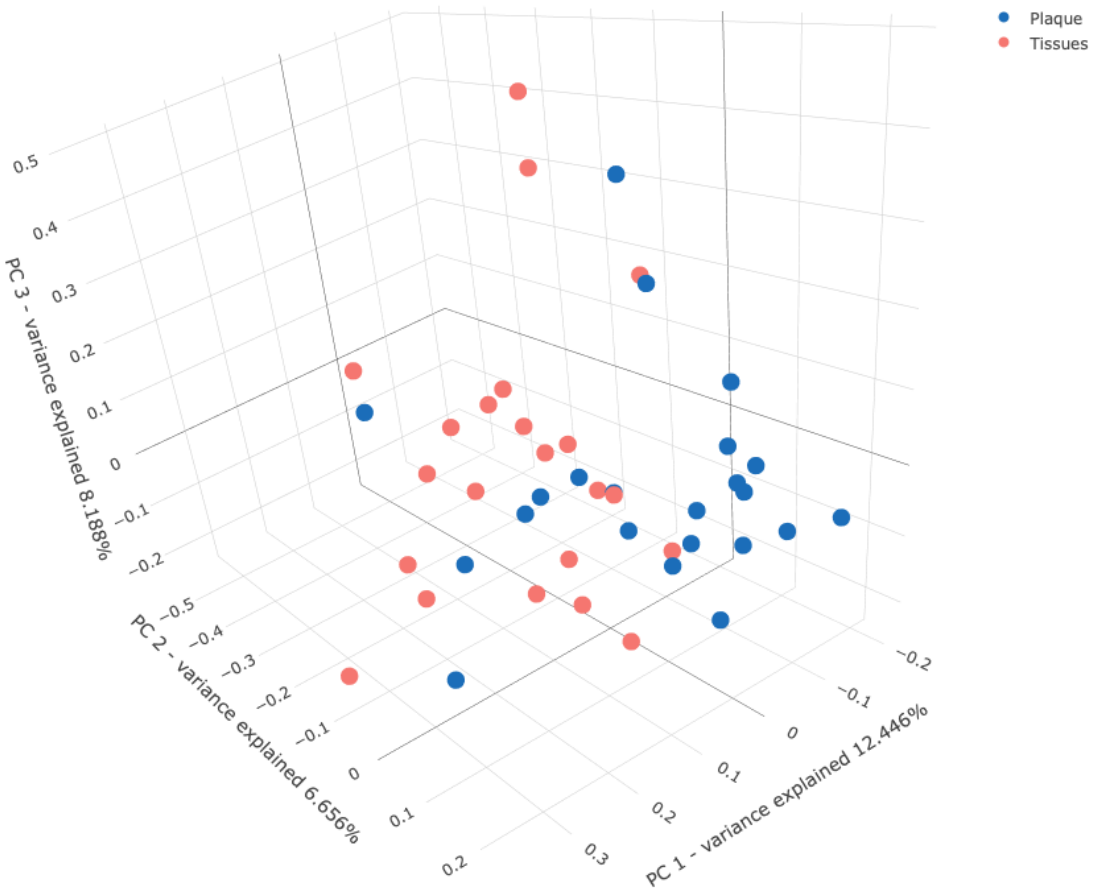

Figure 10: Tensor-based dimensionality reduction for beta diversity. Here each dot represents the microbial changes across the 4 time points. Plaque samples colored red, and tissue samples colored green. The global microbial profile measured by the group centroids were statistically different from each other ($p<0.05$, ADONIS).

To further understand this trajectory, we constructed a Phylogenetic Isometric Log-Ratio (PhILR) transformation (Silverman et al., 2017) for global view of the temporal shifts in microbial composition within subgingival plaque and gingival tissues **(Figure 11)**.

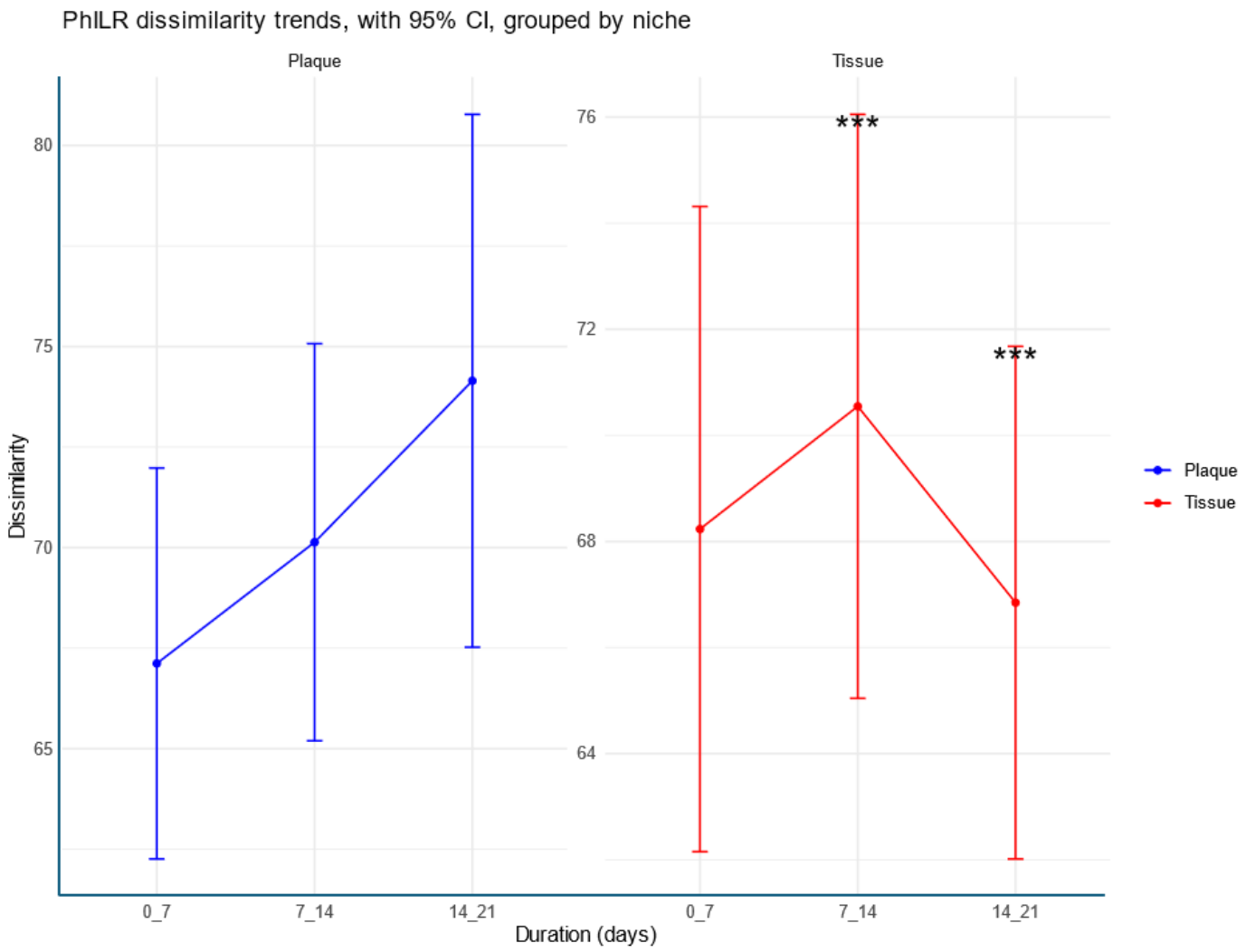

Figure 11: Beta diversity analysis using Phylogenetic Isometric Log-Ratio (PhILR) transformation. The x-axis demonstrates the change in the composition between the four time points: control and day 7 (0_7), between day 7 and day 14 (7_14) and between day 14 and 21 (14_21). The y-axis is the dissimilarity between the different time points as measured by PhILR. The subgingival plaque timeline was colored blue (left) and internalized bacteria within gingival tissues was colored red (right). Graph illustrates the median (colored circle) and the 95% confidence interval (the whiskers) of the dissimilarity. The asterisks (***) illustrate the two time points that were statistically significantly different.

The PhILR transformation revealed distinct temporal changes in the microbiome composition between subgingival plaque and bacteria internalized within gingival tissues (Figure **11**). The composition of the subgingival plaque samples (Figure **11**, left panel) displayed continued increase in dissimilarity within the plaque microbiome, albeit this increase was not statistically significant ($p>0.05$, Friedman's nonparametric rank sum test). In contrast, the dissimilarity in gingival tissue samples **(Figure 11**, right panel) exhibited significant temporal variations in the microbial community composition ($p= 0.04115$, Friedman's nonparametric rank sum test). Nemenyi post-hoc test revealed the decrease in dissimilarity between 7-14 days and 14-21 days to be statistically significant ($p= 0.036$). This significant difference indicates that the maturation of the microbiome in gingival tissues follow a distinct trajectory compared to subgingival plaque. Moreover, this change coincides with the aforementioned statistically significant increase in bleeding on probing, which may indicate that it is part of the normal host response to the gingival tissues that follows changes in the microbiome in the area.

4.3.3 Taxa conserved in plaque and gingival tissues

Next, we wanted to identify whether the compositional changes occurred due to personalized changes in each individual, or whether samples follow some shared patterns. To conduct this, we examined the core taxa in the two niches. The corenesss was set at 60%. That is, for a taxon to be found present, it needed to be seen in 60% of the samples throughout the study. This is a stringent criterion to ensure the conserved taxa's reliability and relevance and to distinguish the core microbiome from transient taxa. Figure 12A shows that plaque harbours a larger microbial core community (blue nodes) compared to gingival tissues (red nodes), underscoring distinct and selective microbial compositions influenced by unique ecological and immunological conditions. This selectivity likely reflects the specific environmental pressures of gingival tissues, impacting which taxa can persist and thrive. Figure 12B identifies taxa common to both environments, suggesting some level of microbial exchange or ecological commonality, although the smaller number of shared taxa further emphasizes the distinct microbial communities in plaque and gingival tissues.

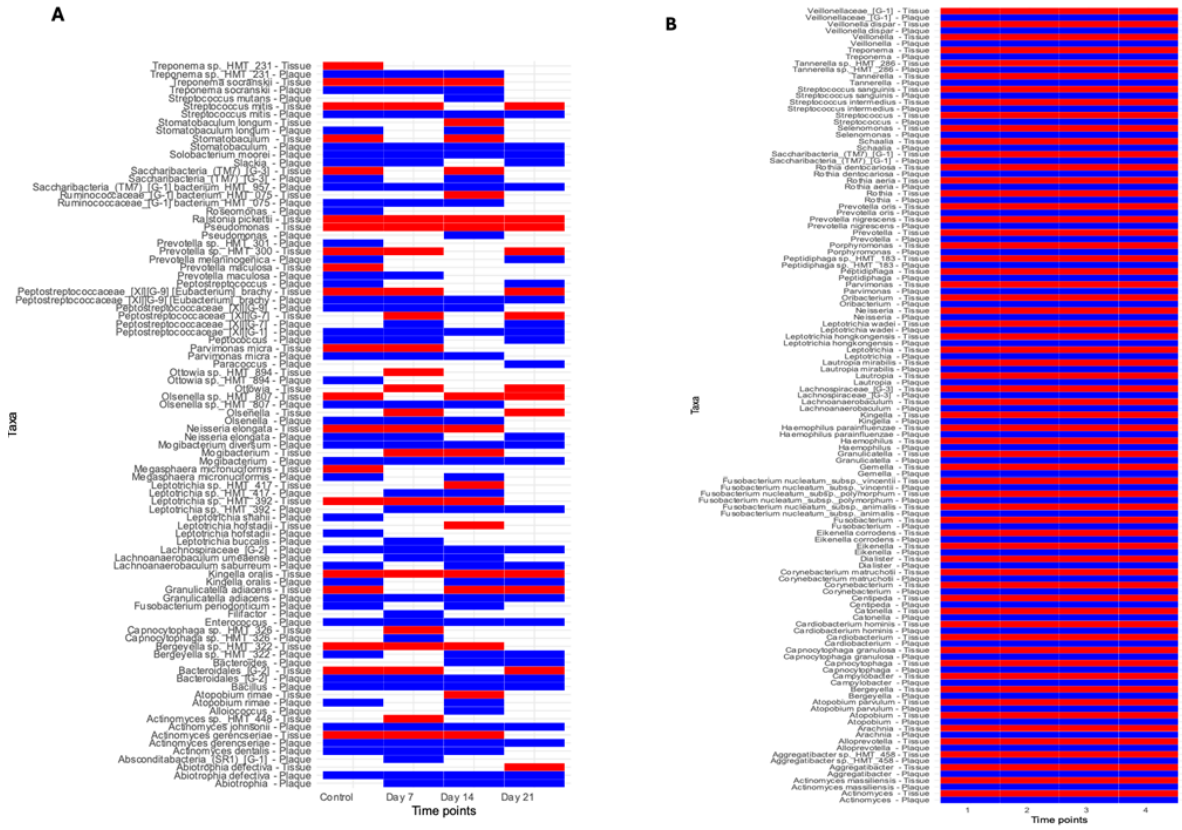

Figure 12: Analysis of core taxa conserved in subgingival plaque and internalized within gingival tissues. The core taxa were defined as those present in at least 60% of the samples. A. The figure shows taxa present in plaque (blue nodes) and gingival tissues (red nodes). The analysis reveals a higher number of taxa in plaque compared to gingival tissues. This indicates that the plaque harbors a more diverse microbial community. In contrast, the gingival tissues exhibit a smaller core, underscoring the environmental pressures exhibited in this niche. B. This panel identifies the taxa that are common to both plaque and gingival tissues

4.3.4: Identifying fluctuations in bacterial taxa during experimental gingivitis.

Figure 13: Mean relative abundance in subgingival plaque, and gingival tissues. These species made up at least a mean of 60% of the species in each group.

Given that subgingival plaque and gingival tissues represent two different environmental niches that require different microbial virulence factors and nutritional/functional capabilities to survive, we wanted to illustrate the largest contributors to each time point (Figure 13). Both niches represent a collection of bacterial members and compositions. Since the progression of experimental gingivitis is accompanied by dynamic changes in the microbial communities within both subgingival plaque and gingival tissues, we set out to test the differences in differential abundances across the different time points and niches.

Several species were statistically significant in all the time points. To reduce the probability of false positives, we removed statistically significant taxa found in less than 50% of the samples, as these may represent aberrant data points that are statistically significant because of their rare presence rather than actual differences in abundance. As such, 7, 10, and 10 species were statistically significantly different between plaque Control/Day 7, Day 7/Day 14, and Day 14/Day 21 samples (Figure 14-16). In contrast, 3, 7 and 2 species were statistically significantly different between gingival tissue Control/Day 7, Day 7/Day 14, and Day 14/Day 21 samples (Figure 17-19). Notably, plaque *Leptrotrichia* and *Prevotella Spps.* decreased in the consecutive time points, with *Leptrotrichia spp.* then rebounding in day 21. A larger difference was seen between plaque and gingival tissues across the different time points (tables 6-9).

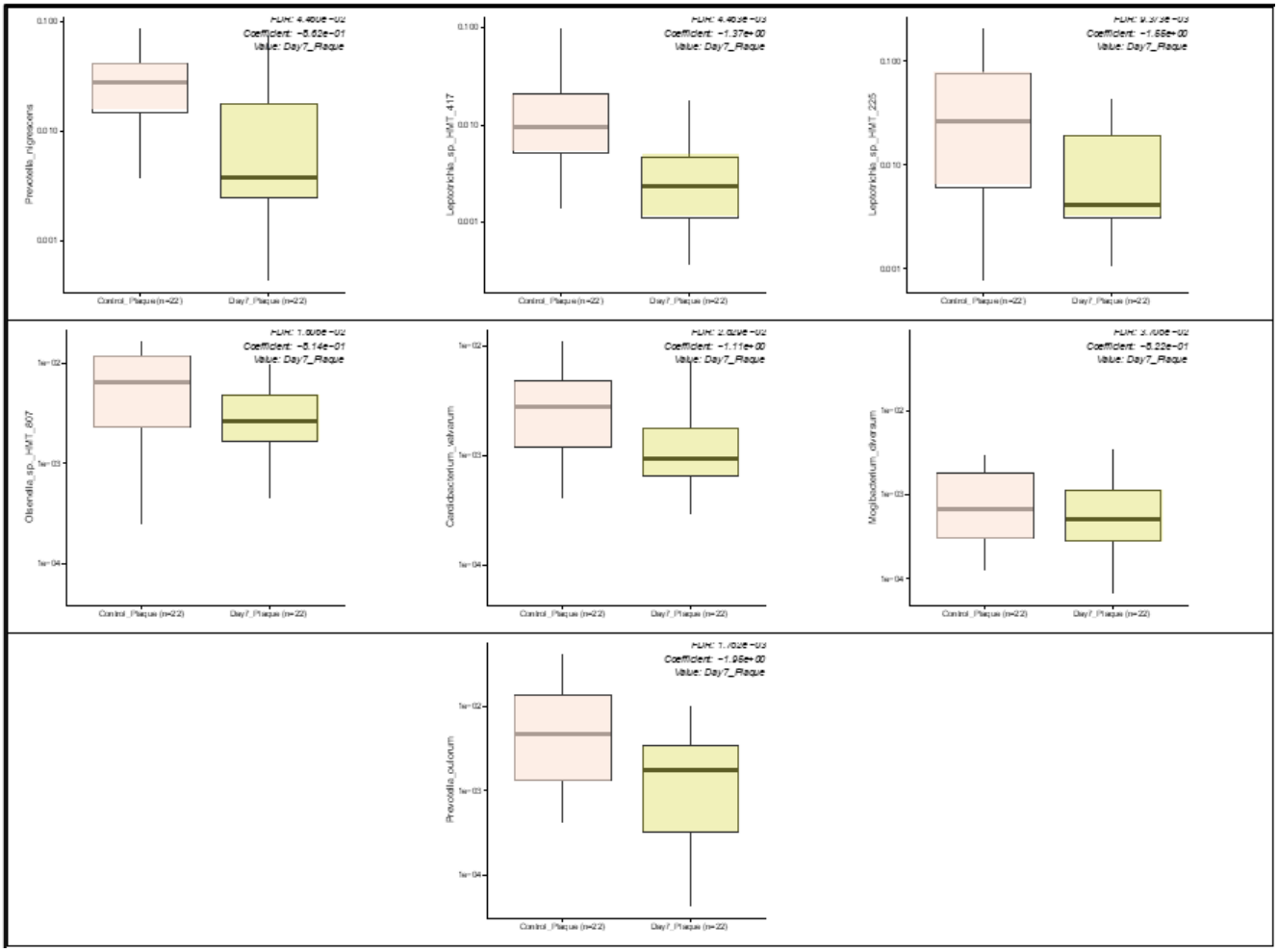

Figure 14: Species that were statistically significantly different from the Control and the Day 7 time points in plaque samples (ZICP, p<0.05). FDR-adjusted pvalues are reported**.**

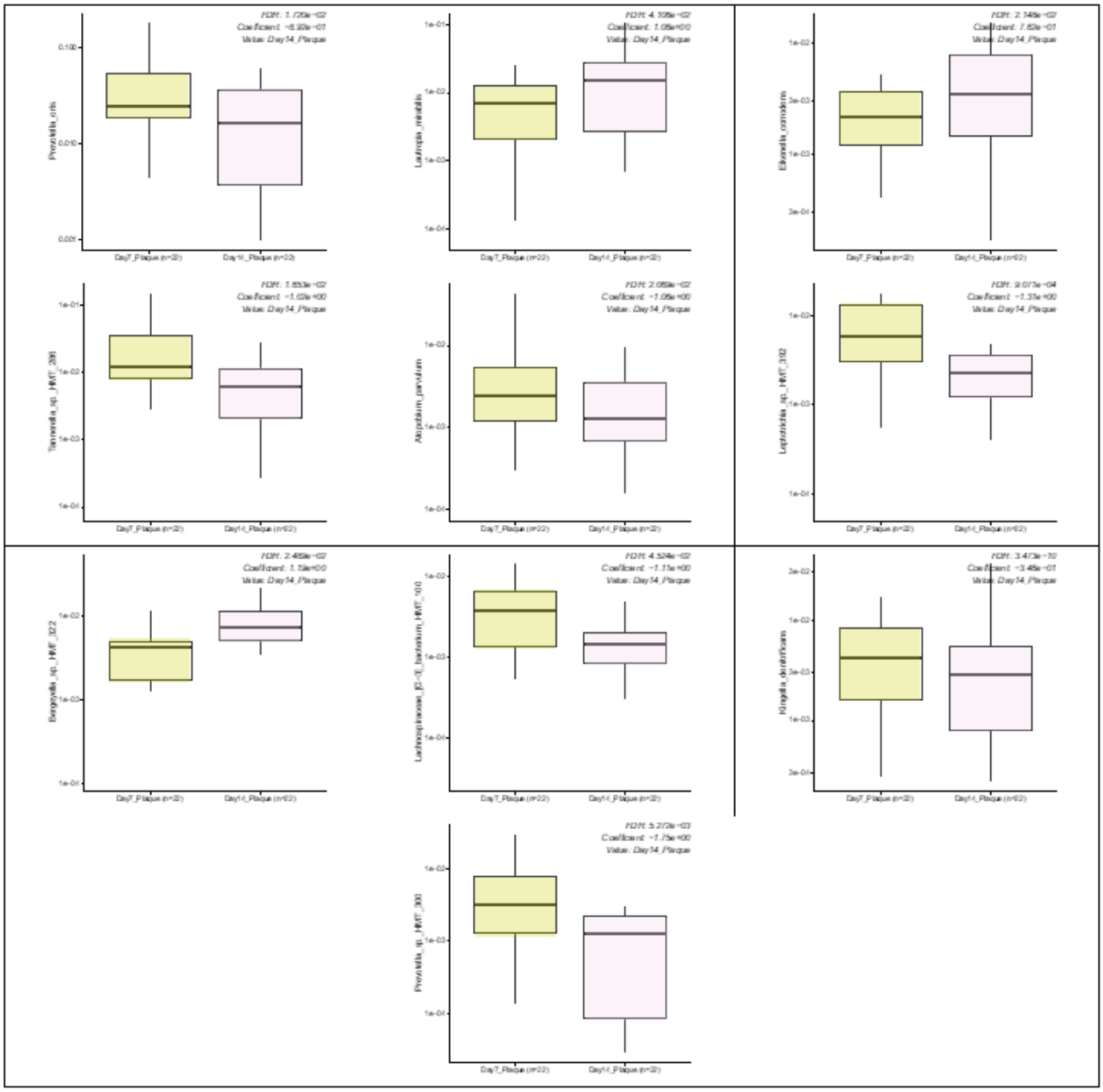

Figure 15: Species that were statistically significantly different from the Day 7 and the Day 14 time points in plaque samples (ZICP, p<0.05). FDR-adjusted pvalues reported

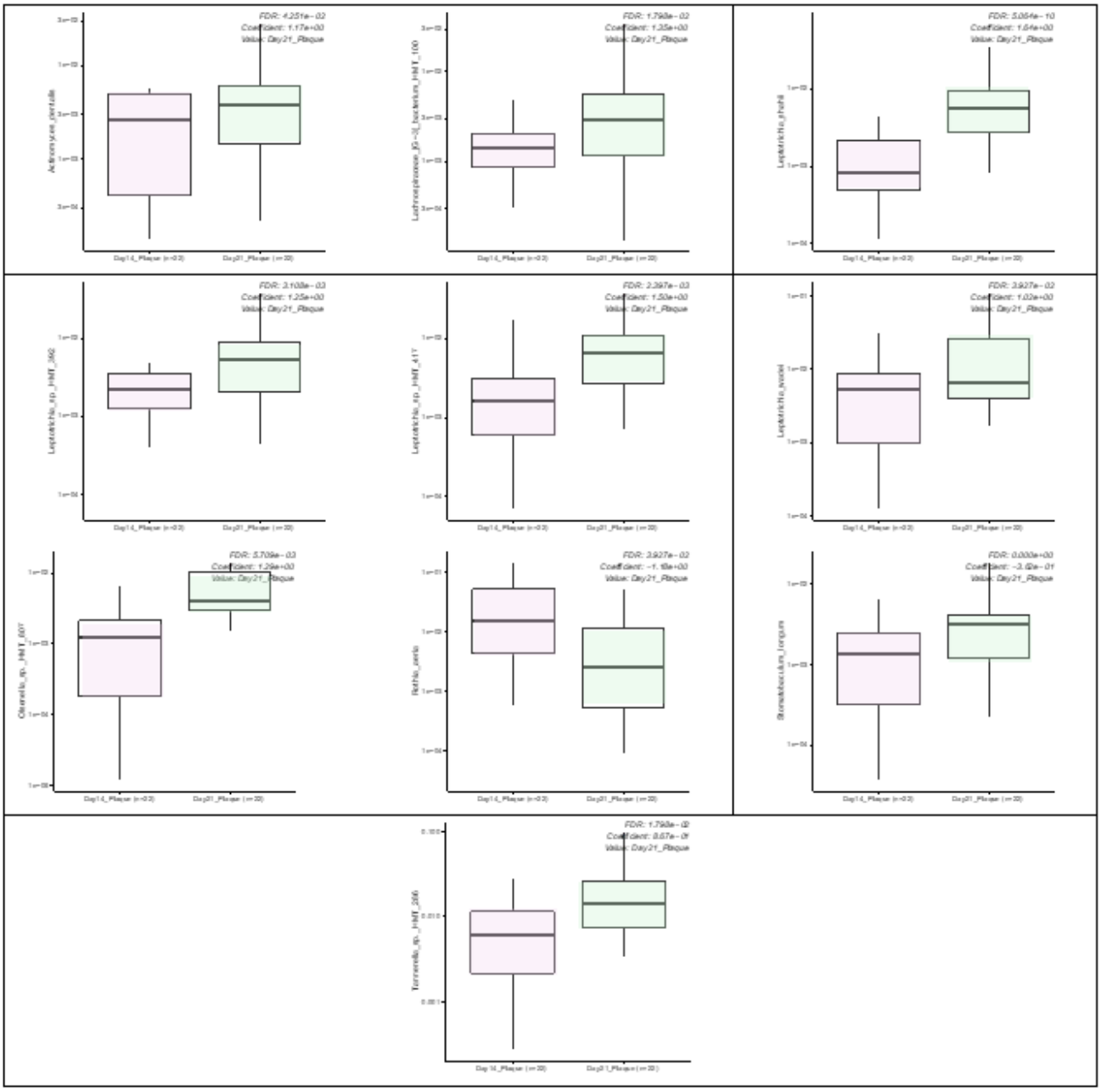

Figure 16: Species that were statistically significantly different from the Day 14 and the Day 21 time points in plaque samples (ZICP, p<0.05). FDR-adjusted pvalues reported

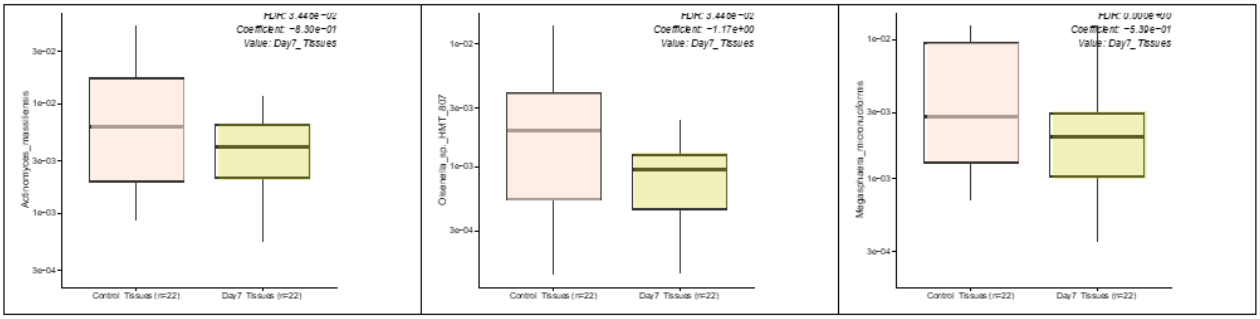

Figure 17: Species that were statistically significantly different from Control and the Day 7 time points in tissue samples (ZICP, p<0.05). FDR-adjusted pvalues reported

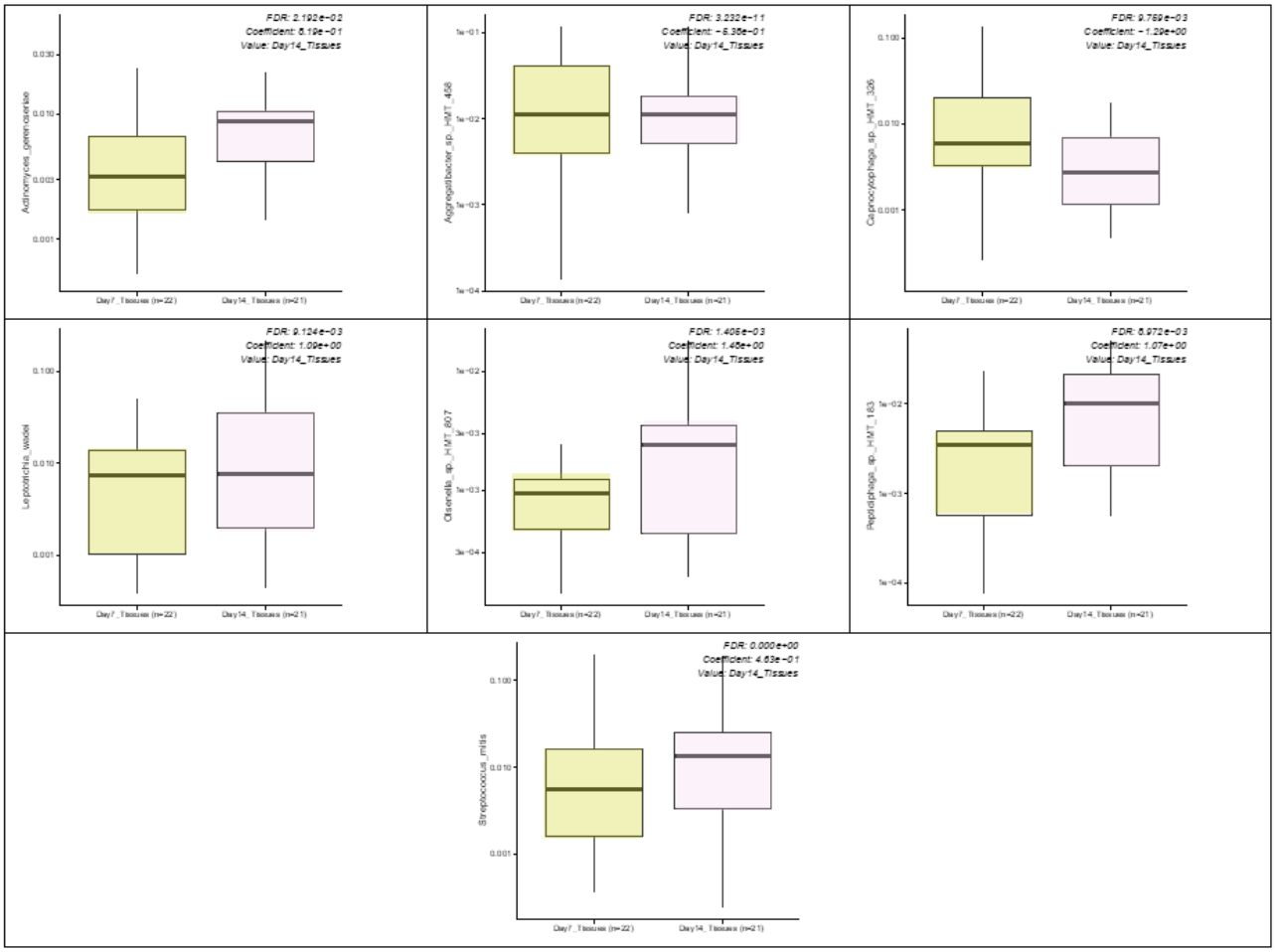

Figure 18: Species that were statistically significantly different from Day 7-14 in tissue samples (ZICP, p<0.05). FDR-adjusted pvalues are reported

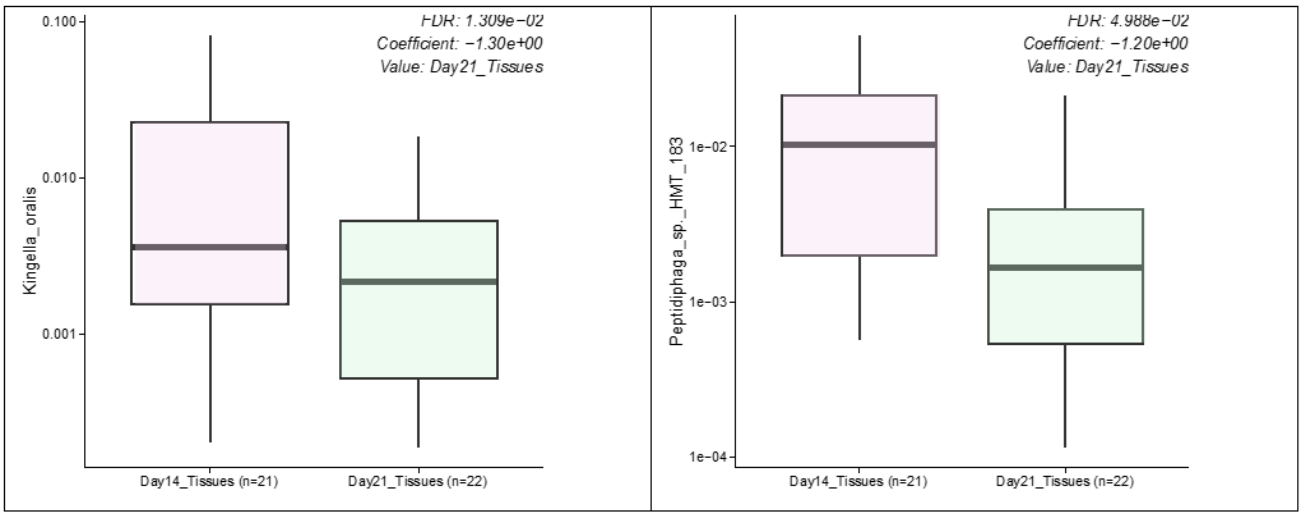

Figure 19: Species that were statistically significantly different from Day 14-21 in tissue samples (ZICP, p<0.05). FDR-adjusted pvalues reported

Comparison	Feature	Coefficient	Std. error	Adj. P value
Control Plaque versus Control Tissues	*Veillonella dispar*	-0.654116628	0.20704397	0.00536139
	Streptococcus sanguinis	-0.840338298	0.20874667	0.00026327
	Streptococcus intermedius	-1.112092419	0.217047526	1.81E-06
	Granulicatella adiacens	-0.794562165	0.211331233	0.00069522
	Fusobacterium nucleatum subsp. vincentii	-0.427012342	0.179439431	0.04014008
	Corynebacterium matruchotii	0.681182336	0.266592519	0.0273222
	Peptostreptococcaceae [XI][G-9] [Eubacterium] brachy	-0.545883192	0.215005232	0.02759948
	Treponema socranskii	0.914891728	0.297847191	0.00704454
	Olsenella sp. HMT 807	-1.056244352	0.372413098	0.01350117
	Ruminococcaceae [G-1] bacterium HMT 075	-0.500328969	0.208669834	0.03942489
	Streptococcus mitis	-0.694541236	0.262363032	0.02255934
	Prevotella sp. HMT 300	1.399611146	0.32770502	0.00010021
	Stomatobaculum longum	-1.287173082	0.43798729	0.01017624
	Kingella denitrificans	-0.500221377	0.019250279	7.79E-148

Table 6: Species statistically different in control samples in plaque and tissue (ZICP, p<0.05). FDR-adjusted p-values and coefficients are reported. Species color indicates higher presence in plaque (blue) or gingival tissues (red).

Comparison	Feature	Coefficient	Std. error	Adj. P value
Day 7 Plaque versus Day 7 Tissues	*Streptococcus intermedius*	-0.901788232	0.273866818	0.00337737
	Rothia dentocariosa	0.661101446	0.288544	0.04905339
	Cardiobacterium hominis	0.750079682	0.197387293	0.00062691
	Rothia aeria	0.894221676	0.267484916	0.00303805
	Prevotella nigrescens	-0.973839625	0.412301665	0.04192818
	Peptostreptococcaceae [XI][G-9] [Eubacterium] brachy	-0.705378528	0.234851702	0.00812022
	Actinomyces gerencseriae	-0.741109622	0.255169992	0.01073918
	Olsenella sp. HMT 807	-1.810314392	0.381956043	1.06E-05
	Ruminococcaceae [G-1] bacterium HMT 075	-1.179069674	0.323047682	0.00110368
	Leptotrichia buccalis	-0.058756622	0.025397437	0.04697645
	Actinomyces sp. HMT 448	-0.510628276	0.000114741	0
	Mogibacterium diversum	-1.564151114	0.331467744	1.13E-05
	Lachnoanaerobaculum umeaense	-0.826427299	0.301433767	0.01618828

Table 7: Species statistically different in day 7 samples in plaque and tissue (ZICP, $p<0.05$). FDR-adjusted p-values and coefficients are reported. Species color indicates higher presence in plaque (blue) or gingival tissues (red).

Comparison	Feature	Coefficient	Std. error	Adj. P value
Day 14 Plaque versus Day 14 Tissues	*Veillonella dispar*	-0.606194325	0.224079755	0.02003008
	Streptococcus sanguinis	-0.841358031	0.214693454	0.00036393
	Cardiobacterium hominis	0.795268472	0.214439937	0.00080391
	Leptotrichia hongkongensis	0.890150716	0.316688719	0.01516219
	Streptococcus intermedius	-1.310440972	0.289739922	2.94E-05
	Actinomyces gerencseriae	0.274486637	0.073297501	0.00071676
	Peptostreptococcaceae [XI][G-9] [Eubacterium] brachy	-1.556697598	0.36553433	9.25E-05
	Bergeyella sp. HMT 322	0.591878101	0.246751745	0.04191204
	Actinomyces johnsonii	-1.733416349	0.384368162	3.02E-05
	Lachnoanaerobaculum umeaense	-0.76569692	0.18644938	0.00017476
	Atopobium rimae	-1.031235576	0.28482489	0.00110222
	Eikenella corrodens	0.838983899	0.306104506	0.01838463
	Mogibacterium diversum	-0.835482589	0.344497219	0.0398362
	Leptotrichia sp. HMT 215	-0.433096804	6.73E-05	0
	Cardiobacterium valvarum	-0.443488421	0.014278065	6.11E-211

Table 8: Species statistically different in day 14 samples in plaque and tissue (ZICP, p<0.05). FDR-adjusted p-values and coefficients are reported. Species color indicates higher presence in plaque (blue) or gingival tissues (red).

Comparison	Feature	Coefficient	Std. error	Adj. P value
Day 21 Plaque versus Day 21 Tissues	*Veillonella dispar*	-0.598768734	0.156020782	0.00056762
	Streptococcus intermedius	-1.044099713	0.309510704	0.0027154
	Corynebacterium matruchotii	0.786318465	0.050136534	1.48E-54
	Eikenella corrodens	0.500707614	0.13691982	0.00099004
	Peptostreptococcaceae [XI][G-9] [Eubacterium] brachy	-0.908517424	0.316453251	0.01309622
	Abiotrophia defectiva	-1.491220587	0.254507455	2.48E-08
	Streptococcus mitis	-0.962923572	0.289339403	0.00305551
	Ottowia sp. HMT 894	1.552704745	0.466936737	0.00305551
	Mogibacterium diversum	-1.137159575	0.450648513	0.03306131
	Saccharibacteria (TM7) [G-1] bacterium HMT 957	-0.802624522	0.102905231	3.78E-14
	Veillonella denticariosi	-0.480470175	0.044208613	1.10E-26
	Lachnoanaerobaculum umeaense	-0.811565469	0.213107517	0.00061774

Table 9: Species statistically different in day 21 samples in plaque and tissue (ZICP, p<0.05). FDR-adjusted p-values and coefficients are reported. Species color indicates higher presence in plaque (blue) or gingival tissues (red).

4.3.5: Microbial interaction networks and diversity dynamics in plaque and gingival tissues

Following the detailed analysis of temporal changes in microbiome composition using PhILR transformation, we extended our investigation to understand the interactions and directional influences within the microbial communities of subgingival plaque and gingival tissues. To achieve this, we employed Extended Local Similarity Analysis (eLSA), a computational method adept at identifying and quantifying temporal associations and directional interactions in complex datasets(L. C. Xia et al., 2011). Unlike traditional methods limited to compositional data or unable to handle longitudinal data, eLSA excels in analyzing a variety of data types, making it particularly suitable for studying dynamic microbial interactions over multiple time points in both ecological and clinical settings. By first transforming the data into a Robus Centered Log-Ratio (rCLR) for both tissue and plaque samples, we were able to transform the data into the Euclidean space, thereby allowing us to overcome the issue of simplex representation of compositional data, which allowed us to use eLSA.

Figure 20 illustrates the directed network analysis using eLSA, revealing the interactions between core plaque taxa (blue nodes) and tissue taxa (red nodes). Positive interactions are represented by green edges, while negative interactions are indicated by red edges, with statistical significance set at $p<0.05$. The size of each node corresponds to its out-degree, signifying the number of taxa it influences. The network shows a high degree of modularity, indicating that species within the plaque significantly impact the tissue microbiome. This tight modularity suggests robust and specific microbial interactions where plaque-associated taxa exert considerable influence on the microbial communities in gingival tissues.

The results showed that the directional influences were consistent across all time points, meaning that the significant relationships observed initially were maintained throughout the study period. Notably, taxa such as OTU1 and OTU2 exhibited consistent directional interactions, indicating stable ecological relationships. Plaque-associated taxa (blue nodes) predominantly influenced tissue-associated taxa (red nodes). The node size visually represents this influence, where larger nodes have more outgoing interactions, indicating greater influence. Positive interactions (green edges) signify an increase in compositional abundance, while negative interactions (red edges)

denote a decrease. The consistent relationships across all time points suggest that the microbial dynamics established early on remained unchanged, underscoring the stability of these interactions. The findings from network analysis, taken together with the findings from the differences in microbial richness and evenness during the experimental gingivitis phase across sequential time points for both plaque and tissue samples indicate that the tissue environment rapidly became less conducive to a wide range of taxa. This finding suggests a quick adaptation or selective pressure in the tissue environment that affects microbial diversity more drastically than in plaque.

Overall, these analyses highlight the intricate and stable nature of microbial interactions within plaque and gingival tissues, while also revealing significant temporal changes in microbial diversity, particularly within tissue samples during the onset of gingivitis.

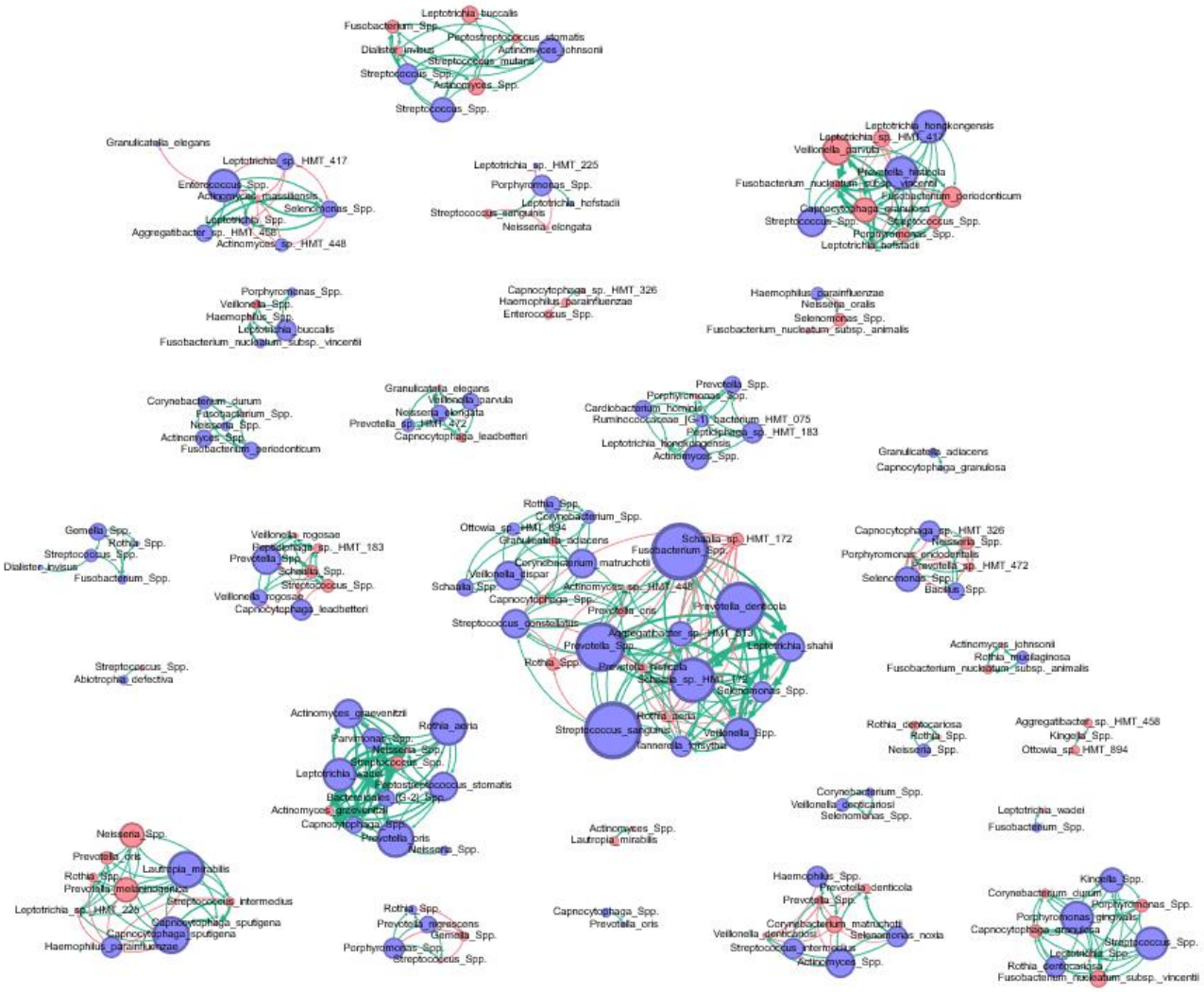

Figure 20: Extended Local Similarity Analysis (eLSA) demonstrating the interactions between core plaque and tissue taxa A directed network analysis using extended Local Similarity Analysis (Elsa) was performed to elucidate the interactions between core plaque taxa (blue nodes) and tissue taxa (red nodes). This analysis captures both positive (green edges) and negative (red edges) interactions, with statistical significance set at $p<0.05$. The size of each node correlates with the out-degree number, indicating the extent to which each taxon influences others within the network.

CHAPTER 5: DISCUSSION

In this study, we characterized the intra-gingival tissue bacterial communities during the induction and resolution of experimental gingivitis in humans. We believe, up to our knowledge, that this is the first time that bacterial internalization into the gingival tissues has been examined under the experimental gingivitis model. The use of this model controls the level of inflammation in the area, as well as the maturation of the subgingival biofilm, which helps us understand the interplay and the differences between the subgingival plaque and gingival tissue microbiome.

The human experimental gingivitis model, introduced in the 1960s, was employed to examine host responses as clinical measures of periodontal health upon cessation of oral hygiene for 21 days. This model was fully reversible to health within 7 days of resuming oral hygiene practices (Loe et al., 1965; Theilade, 1996; Theilade et al., 1966). We used a modified version of this model with partial mouth protocols, rendering each patient as their own control and using an acrylic stent to facilitate oral hygiene cessation. An alternative method would be to sequentially sample the same area after a certain period of oral hygiene cessation. This alternative method has the following disadvantages: 1) longer period of no oral cessation, 2) while multiple sampling from the area has been shown to not significantly affect the subgingival plaque microbiome(Flavia R. Teles, Anne D. Haffajee, & Sigmund S. Socransky, 2008) the same is not known for gingival tissue microbiome, and 3) it is impossible to standardize the environmental exposures that could alter the microbiome in such long experimental periods.

Our split-mouth quasi-longitudinal method ensures that all areas share the environmental effects equally. Our method, however, does not account for site-specific differences in the baseline microbiome in each area. We conducted a scaling and root planing first to help establish a baseline for each area while exposing them to various oral hygiene cessation timelines. Indeed, when we examined the global differences between samples during each time point, we found no statistical difference between the global microbial profiles measured by ADONIS, or between the two using ANOSIM which compares between-group and within-group differences (unpublished results). This could indicate 1) the differences between the groups are small enough not to overcome intergroup differences which indicates the importance of only comparing samples within each patient therefore illustrating the importance of each person's journey and needing to use repeated measures methods, 2) the robust ability of the microbiome to maintain a eubiotic state and recover after short-term disturbances (Almeida et al., 2020; Bamashmous et al., 2021; Deng, Ouyang, Chu,

& Zhang, 2017; Kim et al., 2022; Wirth et al., 2022) and/or 3) the oral hygiene cessation effect was diluted by the microbiota transfer from one area to the other. Indeed, a study that has examined the effect of localized inflammation on distant healthy areas found that the subclinical alterations in proinflammatory host mediators and localized microbial changes within the subgingival microbiome do influence the distant areas in the mouth (Kerns et al., 2023). By eliminating the subgingival plaque in these areas with a professional OH, we do expect oral hygiene cessation to have a stronger effect than the site-specific baseline microbiome. In summary, while our method remains a compromise from a theoretical ideal experimental gingivitis study, we do believe it is better than the sequential method since it provides the best compromise for patient comfort while maintaining an adequate comparison between samples.

To confirm our preliminary hypothesis of bacterial internalization during experimental gingivitis, we used culture-dependent methods to identify bacteria known to internalize into host gingival tissues. The gentamicin/PBS method required that the tissue samples be homogenized after treating with gentamicin to lyse extracellular bacteria, followed by DNAase I treatment to remove any extracellular DNA present in the environment. This means that only inter-and intra-cellular DNA is available for sequencing. The absence of cultures in the gentamycin washing on control plates validated our protocol that no DNA remained outside in the environment. However, quantitative comparisons could not be made due to non-standardized tissue sample weights, and the method's limitation in removing viable bacteria attached in inter-cellular spaces was noted. The selective periodontal pathogens such as *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* and *T. forsythia* that were identified using commercially available culture media demonstrated that these bacteria are indeed present in gingival tissues during the study phases of experimental gingivitis.

Following professional oral hygiene, written and oral hygiene instructions, including the use of a standardized brush and toothpaste without chemotherapeutic aids was provided. Given that the maxilla and mandible may exhibit different clinical presentations due to patient preferences or anatomical differences, we analyzed pre-operative PPD using a linear mixed model approach. No statistically significant differences between the quadrants were found, suggesting effective randomization. This was not unexpected given that we chose patients who are clinically healthy, and that the sites that were chosen were specifically meant to have minimal variations to have a consistent within-subject environment in our quasi-longitudinal style experiment. Interestingly, while no significant differences

were observed in PPD or BOP, we did find that pre-operative plaque levels were significantly higher in the maxilla compared to the mandible ($p = 1.286e-06$, Fisher's exact test). However, considering that plaque was recorded prior to performance of OH, this discrepancy is likely an incidental finding with no substantial clinical relevance to the study.

Next, we assessed for any differences in clinical parameters during the study between baseline, induction, and post-operative periods. Our analysis revealed that while probing pocket depths (PPD) remained stable across the study period, indicating no irreversible periodontal damage, bleeding on probing (BOP) increased significantly during the gingivitis phase and decreased post-intervention, reflecting the inflammatory response to plaque accumulation and its subsequent resolution. The Modified Quigley Hein Plaque Index (MQHPI) showed a similar trend, with increased plaque levels during the induction phase and significant reduction following the resolution phase. Additionally, the papilla, margin, and attachment index demonstrated significant changes; the papilla and margin indices were increased during gingivitis due to inflammation but reverted to baseline levels upon resolution, and the attachment index showed a decrease during inflammation with a subsequent recovery post-intervention. The effect being shown first at the papilla was not a surprising finding, as a similar finding was reported in the classical Løe paper (Loe et al., 1965). As such, in cases were only the papilla was involved, the effect of the microbial invasion would still be captured since the ENAP samples most accurately capture the coronal aspects of the tissues. While the compatibility of subgingival plaque capture with paper points and curettes has been investigated (Belibasakis, Schmidlin, & Sahrmann, 2014), we caution against comparing ENAP to pocket curettage samples, without evidence that the two capture similar microbiome. As such, we suggest this could be a fertile research idea that would help oral researchers standardize our sample capture methods.

We created a repeated-measures-aware distance metric between the samples by creating a tensor that accounts for the day of oral hygiene cessation. The tensor meant that each patient provided one data point for plaque and one for gingival tissues, with the distances representing the trajectory of microbial change in the four time points (**Figure 10**). These two groups' differences allowed us to devise an analysis strategy that would specifically examine this microbial trajectory across the different time points. With the distances from control to day 7 as the baseline, the subgingival plaque had no statistically significant increase in the change in microbial composition across the other time points. Gingival tissues, however, had a statistically significant decrease in microbial dissimilarity between

days 14 and 21, compared to days 7 and 14. We theorize that this is due to the host response to the microbial profile change that most likely lags the changes during the earlier stages of inflammation. As gingival inflammation severity increases, the local environment within the gingival tissues becomes more selective, favouring the proliferation of specific pathogenic species that can survive this increased host inflammatory response, which results in the reduction of overall microbial diversity. This phenomenon has been documented in other studies examining microbial shifts during periodontal disease (Manzoor et al., 2024). The reduction in microbial diversity during disease progression may also reflect the competitive advantage gained by certain pathogens under inflammatory conditions, where dysbiosis induced by inflammation enhances the growth of specific pathogenic bacteria while suppressing commensal species, leading to a more homogenized and pathogenic microbiome.

Due to the large number of core taxa in both subgingival plaque and gingival tissues (**Figure 9**), we suspected these areas would have a predictable recruitment strategy for the microbiota that is conserved across different species based on their phylogenetic similarity (called microbial nepotism), as explained by Dary et al their examination of the human microbiome. While subgingival plaque did follow microbial nepotism, gingival tissues did not. This meant that virulence factors related to microbial fitness in tissue invasion are not phylogenetically conserved. To our knowledge, this is the first human environment that has such a presentation.

The large difference shown in **Figure 10** between subgingival plaque and gingival tissues are in line with studies indicating that the gingival tissues exhibit a unique microbiome composition, influenced by both intrinsic and extrinsic factors (Griffen et al., 2012). This difference encouraged us to examine the two sample types across different time points as our study design gave us the unique opportunity to examine the changes between plaque and gingival tissues at each time point. Many differences in species were found between subgingival plaque and gingival tissues, both qualitatively (coreness in **Figure 12**) and quantitatively (**Tables 6-9** and **Figure 13**). This indicates that the two environments cannot be considered to mirror each other, and that each should be studied as their own separate entity, and to study the effect of one on the other using methods that recognize this directional effect. The microbiome in the subgingival plaque preferentially organizes themselves in predictable structures that may not be reflected in the typical undirected co-occurrence networks (Diaz & Valm, 2019). The method we chose, Extended Local Similarity Analysis – eLSA, specifically looks at the effect of species in a directed way. We were encouraged to see that most of the subgingival plaque microbiota

directly influenced the gingival tissue microbiota (graphically shown in **Figure 17** with the size of the node reflecting the number of connections coming out from it). This reflects the expected physical reality: subgingival plaque microbiota directs the changes in gingival tissue microbiota. This method, however, gave us distinct modules (groups) in which only specific subgingival plaque microbiome influenced specific gingival tissue microbiome and not the expected "hairball" appearance seen in undirected co-occurrence networks. This greatly simplifies the analysis of such networks, where only a small number of bacteria influence a small number of others. This, again, may reflect the physical reality in which the spatial organization of the subgingival microbiome physically puts a small number of species close to the gingival tissues with the exclusion of others. This method gives us candidate connections can be examined in both microbial organization in biofilms and how that differs in their effect in gingival invasion. Streptococci species had a direct, positive connection to *Porphyromonas gingivalis* in our graph. This may reflect the fact that Streptococci species provide streptococcal antigen I/II molecules that work as also function as adherence molecules by *Porphyromonas gingivalis*'s minor fimbrial antigen (Mfa1) (Roky, Trent, & Demuth, 2020). Although examining each connection would be advantageous, understanding these connections goes beyond this study, especially since there may be third-order, fourth-order or beyond connections that would modify these connections. We encourage researchers to develop further methods that would help us identify the most significant groups of nodes that exert the maximal effect beyond the pairwise differences.

Overall, our study provides a comprehensive analysis of microbial communities' interactions and diversity dynamics in subgingival plaque and gingival tissues. The distinct microbial compositions and the selective environment of gingival tissues highlight the complexity of oral microbiome interactions and their implications for oral health.

CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS

6.1. Strength and limitations of the study:

6.1.1. Strengths:

This study provides a comprehensive analysis of the dynamics of the subgingival microbiome and its interaction with host gingival tissues during the development and resolution of experimental gingivitis, employing a high-throughput approach with 16S rDNA sequencing. The study design allows each patient to serve as their own control through partial mouth protocols and the use of acrylic stents to ensure the cessation of oral hygiene, thereby making it easy for the patient to comply with the study without whole-mouth cessation of oral hygiene measures.

One of the key strengths of this study is the utilization of paired models to understand the interactions between plaque and tissue microbiota within the same person. The data was analyzed using the Human Oral Microbiome Database (HOMD) to identify the taxa. Moreover, the utilization of HOMD provided us with a higher resolution phylogenic tree than what would be possible if we constructed the tree based on the short-reads used in this study, as the HOMD phylogenic tree accounts for the entire 16s sequence through a construct that uses several technologies of short and long reads. Moreover, since HOMD classification uses the entire sequence of the 16s, it provides a stringent and reliable framework for identifying conserved taxa, which help us distinguish fundamental microbial populations from transient or less prevalent taxa, thereby enhancing the robustness of microbial analysis(Dewhirst et al., 2010; Griffen et al., 2012).

The study design, employing both alpha and beta diversity analyses, allows for a detailed assessment of microbial diversity and composition changes over time. Alpha diversity measures, such as the Chao1(Chao, 1984), Inverse Simpson(Simpson, 1949), and Shannon indices(Shannon, 1948), provide insights into species richness and evenness within the microbial communities. This dual approach provides a nuanced understanding of how microbial communities shift in response to induced gingivitis. Additionally, Beta diversity metrics, enhanced by techniques like Phylogenetic Isometric Log-Ratio (PhILR) transformation, offer a nuanced understanding of community structure and phylogenetic relationships(Silverman et al., 2017). The usage of analysis techniques such as extended Local Similarity Analysis (eLSA) (L. C. Xia et al., 2011) also enable precise tracking of microbial interactions and directional influences within these communities.

6.1.2. Limitations:

While unique in its exploration, this pilot study is not without its limitations. The small sample size and lack of homogeneity among participants—most of whom were college-educated graduates—may limit the generalizability of the findings. The large age bracket and the unbalanced male-to-female ratio further contribute to this limitation. These demographic factors could introduce bias, as the oral microbiome may be influenced by age, gender, and socioeconomic status. Despite these constraints, the use of each patient as their own control helps mitigate some variability, enhancing the internal validity of the study.

Additionally, the study design necessitates sampling different areas across multiple time points, as opposed to the classical "same area, multiple time" approach of experimental gingivitis. Although previously shown that multiple sampling by curette yields similar retrieved microbiome therefore it does not completely eliminate the subgingival plaque necessitating the restart of the experiment from day zero(F. R. Teles, A. D. Haffajee, & S. S. Socransky, 2008), the same has not been verified for tissue sampling. This means that alternatives to the classical method needed to be devised, such as our "multiple spots of different times sampled at once". By using this method within the same patient post-prophylaxis, we can better control for environmental effects as all sites have had the same environmental exposure throughout the study. The limitations of this method, however, is that it may introduce potential variability in the tissue sampling process that could affect the results, as well as inherent characteristics within each site that are not present in others. To address this, future studies could implement more precise sampling techniques, or alternative sampling strategies, or use advanced imaging technologies to ensure consistent exploration of the gingival tissues.

Another limitation is our ability to make quantitative comparisons on the "proof of concept" culturing experiment due to non-standardized tissue sample weights and the limitation in the method's ability to remove bacteria attached in inter-cellular spaces. This methodological constraint highlights the need for more refined techniques to accurately quantify bacterial internalization in future studies. While these limitations hinder quantitative comparisons in culturing, one of the strengths of our study is the utilization of compositionally aware techniques that internally standardize the measurements as "parts of a whole", which alleviates the issue of different sampling weights, and sequencing coverage across the different samples. Moreover, the qualitative insights gained in this study are still valuable for understanding the dynamics of the oral microbiome.

Furthermore, the potential for microbial transmission from one local site to another cannot be entirely ruled out. However, based on the design of the study, we expect this distant transmission to 1) be equally applicable to all sites, thus equalizing their effect, 2) have a smaller impact than local effects, otherwise, the microbial community would be identical across all sites, which is not observed, and 3) be a natural phenomenon in the oral cavity irrespective of the experimental gingivitis study design. Therefore, expecting no effect from distant sites is contrary to the natural dynamics this clinical study aims to mimic.

In summary, while this study provides valuable insights into the subgingival microbiome's dynamics during experimental gingivitis, addressing these limitations in future research could enhance the robustness and generalizability of the findings.

6.2 Overall conclusions

This quasi-longitudinal study investigated the dynamic interplay between the subgingival plaque microbiome and the internalized microbiome within gingival tissues during the induction and resolution of experimental gingivitis. Our findings underscore a pivotal role of microbial selection in shaping the progression of gingival inflammation, revealing distinct ecological shifts and interactions within these microbial communities.

A key observation was the differential response of plaque and tissue microbiomes to the cessation of oral hygiene. While plaque demonstrated a resilience in species richness, the gingival tissues exhibited a selective pressure, favoring specific taxa as inflammation progressed. This suggests that the tissue environment becomes increasingly selective under inflammatory conditions, potentially due to host immune responses and altered tissue microenvironments.

Furthermore, the study revealed a fascinating pattern of microbial nepotism in plaque, where phylogenetically related bacteria tended to get recruited with time. This phenomenon, however, was not observed in gingival tissues, indicating that the factors influencing bacterial internalization and survival within tissues are not solely determined by phylogenetic relationships. Instead, specific virulence factors and adaptive mechanisms likely play a crucial role in determining which bacteria can successfully invade and persist within gingival tissues.

The analysis of microbial interactions using eLSA highlighted the significant influence of plaque bacteria on the tissue microbiome. This directional interaction suggests that changes in the plaque community can directly impact the composition of bacteria within gingival tissues, potentially contributing to the inflammatory process. The identification of specific taxa and their interactions provides valuable insights into the potential key players in the development and progression of gingivitis.

Overall, this study provides compelling evidence for the importance of microbial selection and ecological interactions in shaping the oral microbiome during gingival inflammation. The distinct responses of plaque and tissue microbiomes, the absence of microbial nepotism in tissues, and the directional influence of plaque on tissue communities underscore the complexity of host-microbe interactions in periodontal health and disease. These findings have significant implications for understanding the pathogenesis of gingivitis and developing targeted interventions to modulate the oral microbiome and promote periodontal health.

6.3 Future directions

The findings of this study add to the very limited pool of knowledge in the area. Future research possibilities stemming from the present findings are multiple including:

Host immune response and bacterial internalization:

Longitudinal immune profiling: A comprehensive longitudinal analysis of host immune responses in the experimental gingivitis model can be conducted on the host GCF and tissue samples. This would involve measuring cytokine and chemokine levels in gingival crevicular fluid (GCF) and saliva at multiple time points throughout the induction and resolution phases. Correlating these immune profiles with the bacterial internalization data would identify specific immune signatures associated with bacterial invasion and persistence.

Single-cell transcriptomics: Single-cell RNA sequencing can also be performed on host tissues to characterize the transcriptomic profiles of gingival epithelial and immune cells during experimental gingivitis. This would allow for the identification of specific cell types and pathways involved in the host response to bacterial internalization.

Innate immune receptors: Innate immune receptors, such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), are instrumental in recognizing and responding to internalized bacteria. An

assessment of expression and activation of these receptors in gingival tissues and assessing their impact on downstream immune signaling pathways could provide invaluable information on early disease mechanisms.

Adaptive immune responses: The role of adaptive immunity, including T cells and B cells, in the response to bacterial internalization could involve assessing their functional responses to internalized bacteria.

Functional consequences of bacterial internalization:

Function of the epithelial barrier: The epithelial barrier is critical to bacterial internalization. An assessment of integrity and function of the gingival epithelial barrier during the various phases of induction and resolution of experimental gingivitis, can identify factors playing a role in its breakdown. This could involve assessing the expression of proteins involved in maintenance of tight junctions and measuring epithelial permeability in the presence of internalized bacteria.

Signaling of inflammatory pathway: An examination of the intracellular signaling pathways activated by internalized bacteria in gingival epithelial and immune cells could involve assessing the activation of NF-κB, MAPK, and other inflammatory signaling pathways and their impact on the production and release of inflammatory mediators.

Therapeutic implications:

The identification of specific bacterial adhesins, invasins, or host cell signaling pathways involved in bacterial internalization opens avenues for developing novel anti-invasive therapies. These therapies could aim to inhibit bacterial uptake by targeting these molecules or pathways, thereby limiting bacterial invasion and subsequent inflammation. The experimental gingivitis model used in this study provides a valuable platform for testing such therapies in a controlled human setting.

Additionally, this model can be utilized to investigate the potential of immunomodulatory therapies to enhance the host response against internalized bacteria. By administering cytokines, chemokines, or other immune modulators, their efficacy in promoting bacterial clearance and resolving inflammation can be assessed. To complement these in vivo studies, developing in vitro models of gingival epithelium and immune cells would allow for a more mechanistic understanding

of bacterial internalization and host immune responses in a controlled environment, free from the complexities of individual patient variability and extrinsic factors.

Finally, integrating multiple "omics" approaches, such as metagenomics, metatranscriptomics, and metabolomics, can provide a comprehensive understanding of the complex host-microbe interactions during experimental gingivitis. This holistic approach can reveal key molecular signatures and pathways associated with bacterial internalization and host responses, ultimately leading to the development of novel, personalized strategies for the prevention and treatment of gingivitis and periodontitis.

REFERENCES

Abiko, Y., Sato, T., Mayanagi, G., & Takahashi, N. (2010). Profiling of subgingival plaque biofilm microflora from periodontally healthy subjects and from subjects with periodontitis using quantitative real-time PCR. *Journal of periodontal research, 45 3*, 389-395.

Abusleme, L., Hoare, A., Hong, B. Y., & Diaz, P. I. (2021). Microbial Signatures of Health, Gingivitis, and Periodontitis. *Periodontology 2000*. doi:10.1111/prd.12362

Ainamo, J. (1992). Significance of epidemiologic research in the understanding of periodontal disease. *Scand J Dent Res, 100*(1), 39-46. doi:10.1111/j.1600-0722.1992.tb01807.x

Al-Kamel, A., Baraniya, D., Al-Hajj, W. A., Halboub, E., Abdulrab, S., Chen, T., & Al-Hebshi, N. N. (2019). Subgingival microbiome of experimental gingivitis: shifts associated with the use of chlorhexidine and N-acetyl cysteine mouthwashes. *J Oral Microbiol, 11*(1), 1608141. doi:10.1080/20002297.2019.1608141

Albandar, J. M., & Kingman, A. (1999). Gingival recession, gingival bleeding, and dental calculus in adults 30 years of age and older in the United States, 1988-1994. *J Periodontol, 70*(1), 30-43. doi:10.1902/jop.1999.70.1.30

Albandar, J. M., & Rams, T. E. (2002). Risk factors for periodontitis in children and young persons. *Periodontology 2000, 29*(1), 207-222.

Almeida, V. S. M., Azevedo, J., Leal, H. F., Queiroz, A. T. L., da Silva Filho, H. P., & Reis, J. N. (2020). Bacterial diversity and prevalence of antibiotic resistance genes in the oral microbiome. *PLOS ONE, 15*(9), e0239664. doi:10.1371/journal.pone.0239664

Andrian, E., Grenier, D., & Rouabhia, M. (2004). In Vitro Models of Tissue Penetration and Destruction by *Porphyromonas gingivalis*. *Infection and immunity, 72*(8), 4689-4698. doi:10.1128/iai.72.8.4689-4698.2004

Arias-Bujanda, N., Regueira-Iglesias, A., Balsa-Castro, C., Nibali, L., Donos, N., & Tomás, I. (2019). Accuracy of Single Molecular Biomarkers in Saliva for the Diagnosis of Periodontitis: A Systematic Review and Meta-analysis. *Journal of Clinical Periodontology*. doi:10.1111/jcpe.13202

Bachrach, G., Ianculovici, C., Naor, R., & Weiss, E. I. (2005). Fluorescence based measurements of Fusobacterium nucleatum coaggregation and of fusobacterial attachment to mammalian cells. *FEMS Microbiology Letters, 248*(2), 235-240. doi:https://doi.org/10.1016/j.femsle.2005.05.055

Bachtiar, E. W., Bachtiar, B. M., Jarosz, L. M., Amir, L. R., Sunarto, H., Ganin, H., . . . Krom, B. P. (2014). AI-2 of *Aggregatibacter actinomycetemcomitans* inhibits *Candida albicans* biofilm formation. *Frontiers in Cellular and Infection Microbiology, 4*.

Baek, K., Ji, S., & Choi, Y. (2018). Complex Intratissue Microbiota Forms Biofilms in Periodontal Lesions. *J Dent Res, 97*(2), 192-200. doi:10.1177/0022034517732754

Bamashmous, S., Kotsakis, G. A., Kerns, K. A., Leroux, B. G., Zenobia, C., Chen, D., . . . Darveau, R. P. (2021). Human variation in gingival inflammation. *Proc Natl Acad Sci U S A, 118*(27). doi:10.1073/pnas.2012578118

Bao, K., Belibasakis, G. N., Thurnheer, T., Aduse-Opoku, J., Curtis, M. A., & Bostanci, N. (2014). Role of Porphyromonas gingivalis gingipains in multi-species biofilm formation. *BMC Microbiology, 14*, 1-8.

Bastian, M. Gephi: An Open Source Software for Exploring and Manipulating Networks. *International AAAI Conference on ….* Retrieved from http://www.academia.edu/302513/Gephi_An_Open_Source_Software_for_Exploring_and_Manipulating_Networks

Beckwith, T. D., Simonton, F., & Williams, A. (1925). A histologic study of the gum in pyorrhea. *The Journal of the American Dental Association (1922), 12*(2), 129-153.

Belibasakis, G. N., Schmidlin, P. R., & Sahrmann, P. (2014). Molecular microbiological evaluation of subgingival biofilm sampling by paper point and curette. *Apmis, 122*(4), 347-352. doi:10.1111/apm.12151

Belton, C. M., Izutsu, K. T., Goodwin, P. C., Park, Y., & Lamont, R. J. (1999). Fluorescence image analysis of the association between Porphyromonas gingivalis and gingival epithelial cells. *Cellular microbiology, 1*(3), 215-223.

Bonfil, J. J., Fourel, J., & Falabregues, R. (1985). The influence of gingival stimulation on recovery from human experimental gingivitis. *J Clin Periodontol, 12*(10), 828-836. doi:10.1111/j.1600-051x.1985.tb01360.x

Brecx, M. C., Lehmann, B., Siegwart, C. M., Gehr, P., & Lang, N. P. (1988). Observations on the initial stages of healing following human experimental gingivitis. A clinical and morphometric study. *J Clin Periodontol, 15*(2), 123-129. doi:10.1111/j.1600-051x.1988.tb01005.x

Brecx, M. C., Schlegel, K., Gehr, P., & Lang, N. P. (1987). Comparison between histological and clinical parameters during human experimental gingivitis. *J Periodontal Res, 22*(1), 50-57. doi:10.1111/j.1600-0765.1987.tb01539.x

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581-583. doi:10.1038/nmeth.3869

Chao, A. (1984). Non-parametric estimation of the classes in a population. *Scandinavian Journal of Statistics, 11*, 265-270. doi:10.2307/4615964

Chapple, I. L. C., Mealey, B. L., Van Dyke, T. E., Bartold, P. M., Dommisch, H., Eickholz, P., . . . Yoshie, H. (2018). Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *Journal of Periodontology, 89*(S1), S74-S84. doi:https://doi.org/10.1002/JPER.17-0719

Chen, T., Yu, W.-H., Izard, J., Baranova, O. V., Lakshmanan, A., & Dewhirst, F. E. (2010). The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database: The Journal of Biological Databases and Curation, 2010*, baq013. doi:10.1093/database/baq013

Christersson, L. A., Albini, B., Zambon, J. J., Wikesjö, U. M., & Genco, R. J. (1987). Tissue localization of Actinobacillus actinomycetemcomitans in human periodontitis. I. Light, immunofluorescence and electron microscopic studies. *J Periodontol, 58*(8), 529-539. doi:10.1902/jop.1987.58.8.529

Clerehugh, V. (2008). Periodontal diseases in children and adolescents. *British Dental Journal, 204*(8).

Colombo, A., Da Silva, C., Haffajee, A., & Colombo, A. (2007). Identification of intracellular oral species within human crevicular epithelial cells from subjects with chronic periodontitis by fluorescence in situ hybridization. *Journal of periodontal research, 42*(3), 236-243.

Colombo, A. P. V., Paster, B. J., Grimaldi, G., Lourenço, T. G. B., Têva, A., Campos-Neto, A., . . . Stashenko, P. (2017). Clinical and Microbiological Parameters of Naturally Occurring Periodontitis in the Non-Human Primate *Macaca Mulatta. Journal of Oral Microbiology*. doi:10.1080/20002297.2017.1403843

Courtois, G. J., 3rd, Cobb, C. M., & Killoy, W. J. (1983). Acute necrotizing ulcerative gingivitis. A transmission electron microscope study. *J Periodontol, 54*(11), 671-679. doi:10.1902/jop.1983.54.11.671

Darcy, J. L., Washburne, A. D., Robeson, M. S., Prest, T., Schmidt, S. K., & Lozupone, C. A. (2020). A phylogenetic model for the recruitment of species into microbial communities and application to studies of the human microbiome. *The ISME Journal, 14*(6), 1359-1368. doi:10.1038/s41396-020-0613-7

Deng, K., Ouyang, X. Y., Chu, Y., & Zhang, Q. (2017). Subgingival microbiome of gingivitis in Chinese undergraduates. *Chin J Dent Res, 20*(3), 145-152.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., . . . Wade, W. G. (2010). The human oral microbiome. *J Bacteriol, 192*(19), 5002-5017. doi:10.1128/jb.00542-10

Diaz, P. I., & Valm, A. M. (2019). Microbial Interactions in Oral Communities Mediate Emergent Biofilm Properties. *Journal of Dental Research, 99*(1), 18-25. doi:10.1177/0022034519880157

Ebersole, J. L., Holt, S. C., & Delaney, J. E. (2013). Acquisition of Oral Microbes and Associated Systemic Responses of Newborn Nonhuman Primates. *Clinical and Vaccine Immunology*. doi:10.1128/cvi.00291-13

Ebersole, J. L., Kirakodu, S., Chen, J., Nagarajan, R., & González, O. A. (2020). Oral Microbiome and Gingival Transcriptome Profiles of Ligature-Induced Periodontitis. *Journal of Dental Research*. doi:10.1177/0022034520906138

Ebersole, J. L., Kirakodu, S., & González, O. A. (2021). Oral Microbiome Interactions With Gingival Gene Expression Patterns for Apoptosis, Autophagy and Hypoxia Pathways in Progressing Periodontitis. *Immunology*. doi:10.1111/imm.13292

Edwards, Grossman, & Rudney. (2006). *Fusobacterium nucleatum* Transports Noninvasive *Streptococcus cristatus* into Human Epithelial Cells. *Infection and immunity, 74*, 654 - 662.

Egelberg, J. (1965). LOCAL EFFECT OF DIET ON PLAQUE FORMATION AND DEVELOPMENT OF GINGIVITIS IN DOGS. I. EFFECT OF HARD AND SOFT DIETS. *Odontol Revy, 16*, 31-41.

Fives-Taylor, P., Meyer, D., & Mintz, K. (1995). Characteristics of Actinobacillus actinomycetemcomitans invasion of and adhesion to cultured epithelial cells. *Adv Dent Res, 9*(1), 55-62. doi:10.1177/08959374950090011001

Fives-Taylor, P. M., Meyer, D. H., Mintz, K. P., & Brissette, C. (1999). Virulence factors of Actinobacillus actinomycetemcomitans. *Periodontol 2000, 20*, 136-167. doi:10.1111/j.1600-0757.1999.tb00161.x

Francis, M., Gopinathan, G., Foyle, D., Fallah, P., Gonzalez, M., Luan, X., & Diekwisch, T. G. H. (2020). Histone Methylation: Achilles Heel and Powerful Mediator of Periodontal Homeostasis. *Journal of Dental Research, 99*(12), 1332-1340. doi:10.1177/0022034520932491

Fredricks, D. N., Schubert, M. M., & Myerson, D. (2005). Molecular Identification of an Invasive Gingival Bacterial Community. *Clinical Infectious Diseases, 41*(1), e1-e4. doi:10.1086/430824

Giannobile, W. V., Finkelman, R. D., & Lynch, S. E. (1994). Comparison of Canine and Non-Human Primate Animal Models for Periodontal Regenerative Therapy: Results Following a Single Administration of PDGF/IGF-I. *Journal of Periodontology*. doi:10.1902/jop.1994.65.12.1158

Gibson III, F., Yumoto, H., Takahashi, Y., Chou, H.-H., & Genco, C. (2006). Innate immune signaling and Porphyromonas gingivalis-accelerated atherosclerosis. *Journal of Dental Research, 85*(2), 106-121.

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., . . . Leys, E. J. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *Isme j, 6*(6), 1176-1185. doi:10.1038/ismej.2011.191

Haffajee, A., Patel, M., & Socransky, S. (2008). Microbiological changes associated with four different periodontal therapies for the treatment of chronic periodontitis. *Oral microbiology and immunology, 23*(2), 148-157.

Hall, M. W., Wellappuli, N. C., Huang, R. C., Wu, K., Lam, D. K., Glogauer, M., . . . Senadheera, D. B. (2023). Suspension of oral hygiene practices highlights key bacterial shifts in saliva, tongue, and tooth plaque during gingival inflammation and resolution. *ISME Communications, 3*(1), 23. doi:10.1038/s43705-023-00229-5

Han, Y. W., Shi, W., Huang, G. T.-J., Kinder Haake, S., Park, N.-H., Kuramitsu, H., & Genco, R. J. (2000). Interactions between periodontal bacteria and human oral epithelial cells: Fusobacterium nucleatum adheres to and invades epithelial cells. *Infection and immunity, 68*(6), 3140-3146.

Hillam, D. G., & Hull, P. S. (1977). The influence of experimental gingivitis on plaque formation. *J Clin Periodontol, 4*(1), 56-61. doi:10.1111/j.1600-051x.1977.tb01883.x

Holm-Pedersen, P., Agerbaek, N., & Theilade, E. (1975). Experimental gingivitis in young and elderly individuals. *J Clin Periodontol, 2*(1), 14-24. doi:10.1111/j.1600-051x.1975.tb01722.x

Holt, S. C., Ebersole, J., Felton, J., Brunsvold, M., & Kornman, K. S. (1988). Implantation of Bacteroides gingivalis in Nonhuman Primates Initiates Progression of Periodontitis. *Science, 239*(4835), 55-57. doi:doi:10.1126/science.3336774

Inagaki, S., Kuramitsu, H. K., & Sharma, A. (2005). Contact-dependent regulation of a Tannerella forsythia virulence factor, BspA, in biofilms. *FEMS Microbiology Letters, 249*(2), 291-296. doi:10.1016/j.femsle.2005.06.032

Inagaki, S., Onishi, S., Kuramitsu, H. K., & Sharma, A. (2006). Porphyromonas gingivalis vesicles enhance attachment, and the leucine-rich repeat BspA protein is required for invasion of epithelial cells by "Tannerella forsythia". *Infection and immunity, 74*(9), 5023-5028.

Johansson, A., Hänström, L., & Kalfas, S. (2000). Inhibition of *Actinobacillus actinomycetemcomitans* leukotoxicity by bacteria from the subgingival flora. *Oral microbiology and immunology, 15 4*, 218-225.

Jones, C. L., Saxton, C. A., & Ritchie, J. A. (1990). Microbiological and clinical effects of a dentifrice containing zinc citrate and Triclosan in the human experimental gingivitis model. *J Clin Periodontol, 17*(8), 570-574.

Jung, Y. J., Jun, H. K., & Choi, B. K. (2017). *Porphyromonas gingivalis* suppresses invasion of *Fusobacterium nucleatum* into gingival epithelial cells. *J Oral Microbiol, 9*(1), 1320193. doi:10.1080/20002297.2017.1320193

Kajikawa, T., Meshikhes, F., Maekawa, T., Hajishengallis, E., Hosur, K. B., Abe, T., . . . Hajishengallis, G. (2017). Milk Fat Globule Epidermal Growth Factor 8 Inhibits Periodontitis in Non-human Primates and Its Gingival Crevicular Fluid Levels Can Differentiate Periodontal Health From Disease in Humans. *Journal of Clinical Periodontology*. doi:10.1111/jcpe.12707

Kerns, K. A., Bamashmous, S., Hendrickson, E. L., Kotsakis, G. A., Leroux, B. G., Daubert, D. D., . . . McLean, J. S. (2023). Localized microbially induced inflammation influences

distant healthy tissues in the human oral cavity. *Proceedings of the National Academy of Sciences, 120*(41), e2306020120. doi:doi:10.1073/pnas.2306020120

Kesavalu, L., Sathishkumar, S., Bakthavatchalu, V., Matthews, C., Dawson, D., Steffen, M., & Ebersole, J. L. (2007). Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease. *Infect Immun, 75*(4), 1704-1712. doi:10.1128/iai.00733-06

Kim, Y. T., Jeong, J., Mun, S., Yun, K., Han, K., & Jeong, S. N. (2022). Comparison of the oral microbial composition between healthy individuals and periodontitis patients in different oral sampling sites using 16S metagenome profiling. *J Periodontal Implant Sci, 52*(5), 394-410. doi:10.5051/jpis.2200680034

Kinane, D., & Attström, R. (2005). Advances in the pathogenesis of periodontitis. Group B consensus report of the fifth European Workshop in Periodontology. *Journal of Clinical Periodontology, 32*, 130-131.

Kistler, J. O., Booth, V., Bradshaw, D. J., & Wade, W. G. (2013). Bacterial Community Development in Experimental Gingivitis. *PLOS ONE, 8*(8), e71227. doi:10.1371/journal.pone.0071227

Klinge, B., Matsson, L., & Attström, R. (1983). Histopathology of initial gingivitis in humans. A pilot study. *J Clin Periodontol, 10*(4), 364-369. doi:10.1111/j.1600-051x.1983.tb01285.x

Kwon, D. H., Bennett, W., Herberg, S., Bastone, P., Pippig, S., Rodriguez, N. A., . . . Wikesjö, U. M. (2010). Evaluation of an injectable rhGDF-5/PLGA construct for minimally invasive periodontal regenerative procedures: a histological study in the dog. *J Clin Periodontol, 37*(4), 390-397. doi:10.1111/j.1600-051X.2010.01546.x

Lai, P. C., & Walters, J. D. (2013). Azithromycin kills invasive Aggregatibacter actinomycetemcomitans in gingival epithelial cells. *Antimicrob Agents Chemother, 57*(3), 1347-1351. doi:10.1128/aac.02558-12

Lamont, R. J., Chan, A., Belton, C. M., Izutsu, K. T., Vasel, D., & Weinberg, A. (1995). Porphyromonas gingivalis invasion of gingival epithelial cells. *Infection and immunity, 63*(10), 3878-3885.

Lang, N. P., & Bartold, P. M. (2018). Periodontal health. *J Periodontol, 89 Suppl 1*, S9-s16. doi:10.1002/jper.16-0517

Lang, N. P., Kiel, R. A., & Anderhalden, K. (1983). Clinical and microbiological effects of subgingival restorations with overhanging or clinically perfect margins. *J Clin Periodontol, 10*(6), 563-578. doi:10.1111/j.1600-051x.1983.tb01295.x

Lima, F. L. d., Lima, F. L. d., Farias, F. F., Costa, J. E., Carvalho, M. A. R., Alviano, C. S., & Farias, L. d. M. (2002). Bacteriocin production by *Actinobacillus actinomycetemcomitans* isolated from the oral cavity of humans with periodontal disease, periodontally healthy subjects and marmosets. *Research in microbiology, 153 1*, 45-52.

Listgarten, M. A. (1965). Electron microscopic observations on the bacterial flora of acute necrotizing ulcerative gingivitis. *J Periodontol (1930), 36*, 328-339. doi:10.1902/jop.1965.36.4.328

Listl, S., Galloway, J., Mossey, P. A., & Marcenes, W. (2015). Global Economic Impact of Dental Diseases. *J Dent Res, 94*(10), 1355-1361. doi:10.1177/0022034515602879

Loe, H., Theilade, E., & Jensen, S. B. (1965). EXPERIMENTAL GINGIVITIS IN MAN. *J Periodontol (1930), 36*, 177-187. doi:10.1902/jop.1965.36.3.177

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., & Knight, R. (2010). UniFrac: an effective distance metric for microbial community comparison. *The ISME Journal, 5*(2), 169-172. doi:10.1038/ismej.2010.133

Mallick, H., Chatterjee, S., Chowdhury, S., Chatterjee, S., Rahnavard, A., & Hicks, S. C. (2022). Differential expression of single-cell RNA-seq data using Tweedie models. *Statistics in Medicine, 41*(18), 3492-3510. doi:https://doi.org/10.1002/sim.9430

Manor, A., Lebendiger, M., Shiffer, A., & Tovel, H. (1984). Bacterial invasion of periodontal tissues in advanced periodontitis in humans. *Journal of Periodontology, 55 10*, 567-573.

Manzoor, M., Leskelä, J., Pietiäinen, M., Martinez-Majander, N., Könönen, E., Niiranen, T., . . . Paju, S. (2024). Shotgun metagenomic analysis of the oral microbiome in gingivitis: a nested case-control study. *Journal of Oral Microbiology, 16*(1), 2330867. doi:10.1080/20002297.2024.2330867

Marchesan, J., Girnary, M. S., Jing, L., Miao, M. Z., Zhang, S., Sun, L., . . . Jiao, Y. (2018). An experimental murine model to study periodontitis. *Nat Protoc, 13*(10), 2247-2267. doi:10.1038/s41596-018-0035-4

Martino, C., Morton, J. T., Marotz, C. A., Thompson, L. R., Tripathi, A., Knight, R., & Zengler, K. (2019). A Novel Sparse Compositional Technique Reveals Microbial Perturbations. *mSystems, 4*(1). doi:10.1128/mSystems.00016-19

Martins, M. D., Jiao, Y., Larsson, L., Almeida, L. O., Garaicoa-Pazmino, C., Le, J. M., . . . Castilho, R. M. (2016). Epigenetic Modifications of Histones in Periodontal Disease. *Journal of Dental Research, 95*(2), 215-222. doi:10.1177/0022034515611876

Mason, M. (2019). *Microbiological analysis of a humanized oral microbiome murine model.* (Masters Thesis). Available from UNC-Chapel Hill Carolina Digital Repository database.

McDonald, D., Vázquez-Baeza, Y., Koslicki, D., McClelland, J., Reeve, N., Xu, Z., . . . Knight, R. (2018). Striped UniFrac: enabling microbiome analysis at unprecedented scale. *Nature Methods, 15*(11), 847-848. doi:10.1038/s41592-018-0187-8

Medapati, M. R., Singh, N., Bhagirath, A. Y., Duan, K., Triggs-Raine, B., Batista, E. L., Jr., & Chelikani, P. (2021). Bitter taste receptor T2R14 detects quorum sensing molecules from cariogenic Streptococcus mutans and mediates innate immune responses in gingival epithelial cells. *Faseb j, 35*(3), e21375. doi:10.1096/fj.202000208R

Mombelli, A., Lang, N. P., Bürgin, W. B., & Gusberti, F. A. (1990). Microbial changes associated with the development of puberty gingivitis. *J Periodontal Res, 25*(6), 331-338. doi:10.1111/j.1600-0765.1990.tb00924.x

Moore, W. E., Holdeman, L. V., Smibert, R. M., Good, I. J., Burmeister, J. A., Palcanis, K. G., & Ranney, R. R. (1982). Bacteriology of experimental gingivitis in young adult humans. *Infect Immun, 38*(2), 651-667. doi:10.1128/iai.38.2.651-667.1982

Morelli, T., Stella, M., Barros, S. P., Marchesan, J. T., Moss, K., Kim, S. J., . . . Offenbacher, S. (2014). Salivary Biomarkers in a Biofilm Overgrowth Model. *Journal of Periodontology*. doi:10.1902/jop.2014.140180

Nazir, M. A., Al-Ansari, A., Al-Khalifa, K. S., Alhareky, M., Gaffar, B., & Almas, K. (2020). Global Prevalence of Periodontal Disease and Lack of Its Surveillance. *The Scientific World Journal*. doi:10.1155/2020/2146160

Nowicki, E. M., Shroff, R., Singleton, J. A., Renaud, D. E., Wallace, D., Drury, J., . . . Whiteley, M. (2018). Microbiota and Metatranscriptome Changes Accompanying the Onset of Gingivitis. *mBio, 9*(2). doi:10.1128/mBio.00575-18

Offenbacher, S., Barros, S. P., Paquette, D. W., Winston, J., Biesbrock, A. R., Thomason, R., . . . Ho, B. Y. (2009). Gingival Transcriptome Patterns During Induction and Resolution of Experimental Gingivitis in Humans. *Journal of Periodontology*. doi:10.1902/jop.2009.080645

Oh, T. J., Eber, R., & Wang, H. L. (2002). Periodontal diseases in the child and adolescent. *Journal of Clinical Periodontology, 29*(5), 400-410.

Orth, R. K. H., O'Brien-Simpson, N. M., Dashper, S. G., & Reynolds, E. C. (2011). Synergistic virulence of *Porphyromonas gingivalis* and *Treponema denticola* in a murine periodontitis model. *Molecular Oral Microbiology, 26*(4), 229-240. doi:10.1111/j.2041-1014.2011.00612.x

Oz, H. S., & Puleo, D. A. (2011). Animal Models for Periodontal Disease. *Journal of Biomedicine and Biotechnology*. doi:10.1155/2011/754857

Papapanou, P. N., Behle, J. H., Kebschull, M., Celenti, R., Wolf, D. L., Handfield, M., . . . Demmer, R. T. (2009). Subgingival bacterial colonization profiles correlate with gingival ti ssue gene expression. *BMC Microbiology, 9*(1), 221. doi:10.1186/1471-2180-9-221

Pavlica, Z., Petelin, M., Nemec, A., Erzen, D., & Skaleric, U. (2004). Measurement of total antioxidant capacity in gingival crevicular fluid and serum in dogs with periodontal disease. *Am J Vet Res, 65*(11), 1584-1588. doi:10.2460/ajvr.2004.65.1584

Petersen, P. E., & Ogawa, H. (2012). The Global Burden of Periodontal Disease: Towards Integration With Chronic Disease Prevention and Control. *Periodontology 2000*. doi:10.1111/j.1600-0757.2011.00425.x

Putt, M. S., Van der Weijden, G. A., Kleber, C. J., & Saxton, C. A. (1993). Validation of a 21-day, partial-mouth gingivitis model for evaluating chemotherapeutic dentifrices. *J Periodontal Res, 28*(4), 301-307. doi:10.1111/j.1600-0765.1993.tb02097.x

Quigley, G. A., & Hein, J. W. (1962). Comparative cleansing efficiency of manual and power brushing. *The Journal of the American Dental Association, 65*(1), 26-29.

Rojas, C., García, M. P., Polanco, A. F., González-Osuna, L., Sierra-Cristancho, A., Melgar-Rodríguez, S., . . . Vernal, R. (2021). Humanized Mouse Models for the Study of Periodontitis: An Opportunity to Elucidate Unresolved Aspects of Its Immunopathogenesis and Analyze New Immunotherapeutic Strategies. *Frontiers in Immunology, 12*. doi:10.3389/fimmu.2021.663328

Roky, M., Trent, J. O., & Demuth, D. R. (2020). Identification of functional domains of the minor fimbrial antigen involved in the interaction of Porphyromonas gingivalis with oral streptococci. *Molecular Oral Microbiology, 35*(2), 66-77. doi:https://doi.org/10.1111/omi.12280

Rudney, J., Chen, R., & Sedgewick, G. (2005). Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, and Tannerella forsythensis are components of a polymicrobial intracellular flora within human buccal cells. *Journal of Dental Research, 84*(1), 59-63.

Rudney, J., Chen, R., & Zhang, G. (2005). Streptococci dominate the diverse flora within buccal cells. *Journal of Dental Research, 84*(12), 1165-1171.

Saglie, F. R., Pertuiset, J. H., Rezende, M. T., Sabet, M. S., Raoufi, D., & Carranza, F. A., Jr. (1987). Bacterial Invasion in Experimental Gingivitis in Man. *Journal of Periodontology, 58*(12), 837-846. doi:10.1902/jop.1987.58.12.837

Saglie, R., & Elbaz, J. J. (1983). Bacterial penetration into the gingival tissue in periodontal disease. *J West Soc Periodontol Periodontal Abstr, 31*(3), 85-93.

Saglie, R., Newman, M. G., Carranza, F. A., Jr., & Pattison, G. L. (1982). Bacterial invasion of gingiva in advanced periodontitis in humans. *J Periodontol, 53*(4), 217-222. doi:10.1902/jop.1982.53.4.217

Saito, A., Inagaki, S., & Ishihara, K. (2009). Differential ability of periodontopathic bacteria to modulate invasion of human gingival epithelial cells by Porphyromonas gingivalis. *Microbial Pathogenesis, 47*(6), 329-333. doi:10.1016/j.micpath.2009.09.012

Sandros, J., Papapanou, P., & Dahlén, G. (1993). Porphyromonas gingivalis invades oral epithelial cells in vitro. *J Periodontal Res, 28*(3), 219-226. doi:10.1111/j.1600-0765.1993.tb01072.x

Sandros, J., Papapanou, P. N., Nannmark, U., & Dahlén, G. (1994). Porphyromonas gingivalis invades human pocket epithelium in vitro. *J Periodontal Res, 29*(1), 62-69. doi:10.1111/j.1600-0765.1994.tb01092.x

Savitt, E. D., & Socransky, S. S. (1984). Distribution of certain subgingival microbial species in selected periodontal conditions. *J Periodontal Res, 19*(2), 111-123. doi:10.1111/j.1600-0765.1984.tb00800.x

Scannapieco, F. A. (2004). Periodontal inflammation: from gingivitis to systemic disease? *Compend Contin Educ Dent, 25*(7 Suppl 1), 16-25.

Schmit, V. L., Patton, T. G., & Gilmore, R. D. (2011). Analysis of Borrelia burgdorferi Surface Proteins as Determinants in Establishing Host Cell Interactions. *Frontiers in Microbiology, 2*. doi:10.3389/fmicb.2011.00141

Schou, S., Holmstrup, P., & Kornman, K. S. (1993). Non-Human Primates Used in Studies of Periodontal Disease Pathogenesis: A Review of the Literature. *Journal of Periodontology, 64*(6), 497-508. doi:https://doi.org/10.1902/jop.1993.64.6.497

Schour, I., & Massler, M. (1947). Gingival disease in postwar Italy (1945) prevalence of gingivitis in various age groups. *J Am Dent Assoc, 35*(7), 475-482. doi:10.14219/jada.archive.1947.0266

Seymour, G. J., Powell, R. N., & Aitken, J. F. (1983). Experimental gingivitis in humans. A clinical and histologic investigation. *J Periodontol, 54*(9), 522-528. doi:10.1902/jop.1983.54.9.522

Shannon, C. E. (1948). A mathematical theory of communication. *The Bell system technical journal, 27*(3), 379-423.

Shi, P., Martino, C., Han, R., Janssen, S., Buck, G., Serrano, M., . . . Zhang, A. R. (2024). Time-Informed Dimensionality Reduction for Longitudinal Microbiome Studies. *bioRxiv*, 2023.2007.2026.550749. doi:10.1101/2023.07.26.550749

Silverman, J. D., Washburne, A. D., Mukherjee, S., & David, L. A. (2017). A phylogenetic transform enhances analysis of compositional microbiota data. *eLife, 6*, e21887. doi:10.7554/eLife.21887

Simpson, E. H. (1949). Measurement of diversity. *nature, 163*(4148), 688-688.

Slots, J., Möenbo, D., Langebaek, J., & Frandsen, A. M. (1978). Microbiota of gingivitis in man. *Scandinavian journal of dental research, 86 3*, 174-181.

Socransky, S. S., & Haffajee, A. D. (1991). Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. *J Periodontal Res, 26*(3 Pt 2), 195-212. doi:10.1111/j.1600-0765.1991.tb01646.x

Sreenivasan, P. K., Meyer, D. H., & Fives-Taylor, P. M. (1993). Factors influencing the growth and viability of Actinobacillus actinomycetemcomitans. *Oral microbiology and immunology, 8*(6), 361-369. doi:https://doi.org/10.1111/j.1399-302X.1993.tb00612.x

Syed, S. A., & Loesche, W. J. (1978). Bacteriology of human experimental gingivitis: effect of plaque age. *Infect Immun, 21*(3), 821-829. doi:10.1128/iai.21.3.821-829.1978

Tan, K. H., Seers, C. A., Dashper, S. G., Mitchell, H. L., Pyke, J. S., Meuric, V., . . . Reynolds, E. C. (2014). *Porphyromonas gingivalis* and *Treponema denticola* Exhibit Metabolic Symbioses. *PLoS Pathogens, 10*(3), e1003955. doi:10.1371/journal.ppat.1003955

Teles, F. R., Haffajee, A. D., & Socransky, S. S. (2008). The Reproducibility of Curet Sampling of Subgingival Biofilms. *Journal of Periodontology, 79*(4), 705-713. doi:https://doi.org/10.1902/jop.2008.070424

Teles, F. R., Haffajee, A. D., & Socransky, S. S. (2008). The reproducibility of curet sampling of subgingival biofilms. *J Periodontol, 79*(4), 705-713. doi:10.1902/jop.2008.070424

Theilade, E. (1996). The experimental gingivitis studies: the microbiological perspective. *J Dent Res, 75*(7), 1434-1438. doi:10.1177/00220345960750070201

Theilade, E., Wright, W. H., Jensen, S. B., & Löe, H. (1966). Experimental gingivitis in man. II. A longitudinal clinical and bacteriological investigation. *J Periodontal Res, 1*, 1-13. doi:10.1111/j.1600-0765.1966.tb01842.x

Thiha, K., Takeuchi, Y., Umeda, M., Huang, Y., Ohnishi, M., & Ishikawa, I. (2007). Identification of periodontopathic bacteria in gingival tissue of Japa nese periodontitis patients. *Oral microbiology and immunology, 22*(3), 201-207. doi:10.1111/j.1399-302x.2007.00354.x

Tonetti, M. S., Jepsen, S., Jin, L., & Otomo-Corgel, J. (2017). Impact of the Global Burden of Periodontal Diseases on Health, Nutrition and Wellbeing of Mankind: A Call for Global Action. *Journal of Clinical Periodontology*. doi:10.1111/jcpe.12732

Turesky, S. (1970). Reduced plaque formation by the chloromethyl analogue vitamine C. *J Periodontol, 41*, 41-43.

Turner, J. G., & Drew, A. H. (1919). An Experimental Inquiry into the Bacteriology of Pyorrhoea. *Proc R Soc Med, 12*(Odontol Sect), 104-118.

Vodička, P., Smetana Jr., K., Dvořánková, B., Emerick, T., Xu, Y. Z., Ourednik, J., . . . Motlík, J. (2005). The Miniature Pig as an Animal Model in Biomedical Research. *Annals of the New York Academy of Sciences, 1049*(1), 161-171. doi:https://doi.org/10.1196/annals.1334.015

Wang, S., Liu, Y., Fang, D., & Shi, S. (2007). The miniature pig: a useful large animal model for dental and orofacial research. *Oral Dis, 13*(6), 530-537. doi:10.1111/j.1601-0825.2006.01337.x

Weinberg, M. A., & Bral, M. (1999). Laboratory Animal Models in Periodontology. *Journal of Clinical Periodontology*. doi:10.1034/j.1600-051x.1999.260601.x

Weinstein, M. M., Prem, A., Jin, M., Tang, S., & Bhasin, J. M. (2019). FIGARO: An efficient and objective tool for optimizing microbiome rRNA gene trimming parameters. *bioRxiv*, 610394. doi:10.1101/610394

WHO. (2022). Global oral health status report: towards universal health coverage for oral health by 2030. Retrieved from https://www.who.int/publications/i/item/9789240061484. https://www.who.int/publications/i/item/9789240061484

Wirth, R., Maróti, G., Lipták, L., Mester, M., Al Ayoubi, A., Pap, B., . . . Kovács, K. L. (2022). Microbiomes in supragingival biofilms and saliva of adolescents with gingivitis and gingival health. *Oral Diseases, 28*(7), 2000-2014.

Woelber, J. P., Gärtner, M., Breuninger, L., Anderson, A. B. M., König, D., Hellwig, E., . . . Tennert, C. (2019). The Influence of an Anti-inflammatory Diet on Gingivitis. A Randomized Controlled Trial. *Journal of Clinical Periodontology*. doi:10.1111/jcpe.13094

Wong, R. G., Wu, J. R., & Gloor, G. B. (2016). Expanding the UniFrac Toolbox. *PLOS ONE, 11*(9), e0161196. doi:10.1371/journal.pone.0161196

Xia, L. C., Steele, J. A., Cram, J. A., Cardon, Z. G., Simmons, S. L., Vallino, J. J., . . . Sun, F. (2011). Extended local similarity analysis (eLSA) of microbial community and other time series data with replicates. *BMC Systems Biology, 5*(2), S15. doi:10.1186/1752-0509-5-S2-S15

Xia, L. C., Steele, J. A., Cram, J. A., Cardon, Z. G., Simmons, S. L., Vallino, J. J., . . . Sun, F. (2011). Extended local similarity analysis (eLSA) of microbial community and other time series data with replicates. *BMC Syst Biol, 5 Suppl 2*(Suppl 2), S15. doi:10.1186/1752-0509-5-s2-s15

Yates, R. J., Shearer, B., Morgan, R., & Addy, M. (2003). A Modification to the Experimental Gingivitis Protocol to Compare the Antiplaque Properties of Two Toothpastes. *Journal of Clinical Periodontology*. doi:10.1034/j.1600-051x.2003.00205.x

Yilmaz, Ö., Watanabe, K., & Lamont, R. J. (2002). Involvement of integrins in fimbriae-mediated binding and invasion by Porphyromonas gingivalis. *Cellular microbiology, 4*(5), 305-314.

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

Yukna, R. A. (1978). Longitudinal evaluation of the excisional new attachment procedure in humans. *J Periodontol, 49*(3), 142-144. doi:10.1902/jop.1978.49.3.142

Zemouri, C., Jakubovics, N. S., Zaura, E., Dodds, M., Schelkle, B., & Loos, B. G. (2019). Resistance and Resilience to Experimental Gingivitis: A Systematic Scoping Review. *BMC Oral Health*. doi:10.1186/s12903-019-0889-z