Changes in a Child's Subgingival Microbiome Following Prophylaxis – a Pilot Study

by

Andrew Gibb

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

Master of Science

Medical Sciences - Periodontology
University of Alberta

THESIS ABSTRACT

Background:

Periodontitis is a multifactorial, complex chronic oral disease facilitated by oral microbes. Transmissibility studies of the past have shown that oral microbes are transmissible between individuals. Furthermore, periodontal disease phenotypes also appear to be inheritable to some extent, both from parent to child and between adults. Modern DNA sequencing has allowed for greater resolution and further insight into the transmission rates of the oral microbiome. In this study, transmission rates of the oral microbiome were investigated within the family unit, especially as it relates to the recolonization of the oral microbiome of the youngest child in multi-child families.

Aims: 1) Identify the sources of subgingival microbiome within an individual. 2) Identify the effect of intimate contact between parents and child as well as between siblings and child in vertical and horizontal transmission of the microbiome. 3) Examine the effect of recolonization on the child after professional oral prophylaxis.

Materials and methods: 14 families were recruited, each family having 1 mother, 1 father, and two or more children. Microbial samples were collected from each individual. Oral prophylaxis was then performed for the youngest child. An additional saliva sample was collected from the youngest child at day 3, and then all oral samples again collected for the youngest child at one week following prophylaxis. Samples were then processed and DNA isolated, sequenced (V1-V3), and passed through a computational pipeline (DADA2) to determine amplicon sequence variants (ASVs). These were then analyzed using a Bayesian analysis model to estimate the contribution of all samples to the subgingival microbiota of the youngest child.

Results: The findings of this study are four-fold:

1. Oral niche was the main driver of microbial separation. The microbes that were found in a specific location in the mouth were mainly selected due to the characteristics of that niche rather than by familial similarity or geographical proximity.
2. Saliva was the conduit for oral cavity microbiota. Saliva was the main vehicle for microbial transmission between individuals. The saliva was made up of microbes from other areas of the mouth, including the tongue (58.9%), buccal mucosa (14.8%), supragingival plaque (1.1%), subgingival plaque (0.8%), and unknown sources (12.5%).
3. Various sources of microbes contributed to the subgingival plaque of the child. Some of these sources were internal to the child, specifically the subgingival plaque already present at time 0 (46%), as well as the supragingival plaque at time 0 (9%), and buccal mucosal microbes (1%). Sources external to the child also existed. Both mother and father contributed about 3% each to the recolonization of the oral microbiome, while each sibling contributed approximately 8%. Of those sources external to the child, 8% was from subgingival sources, 4% from supragingival sources, and 1% was from the buccal. All other sources were negligible.
4. The only close contact activities that had a statistically significant contribution to the subgingival plaque of the child included mother close sleeping with the child and saliva contribution, and father kissing the child on the cheek and buccal mucosal microbial transmission.

Conclusion: Within the family unit, transmission of oral microbes does occur. This transmission is most pronounced between siblings (8%), and not as strongly from parent to child (3%) as originally expected. The majority of this transmission is via the saliva, which contains only 0.8% of subgingival bacteria. Even then, the major contributor to the subgingival microbes of the child from external sources is the subgingival bacteria of family members. As shown in this study, transmission of oral microbes between family members does occur, with greater importance seen in the contribution of siblings than expected. Thus, the treatment of periodontal disease may need to shift from an individual disease control model to a family-based, transmissible disease model.

PREFACE

This thesis is an original work by Andrew Gibb. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board 3, Project Name “Changes in a child’s subgingival microbiome following prophylaxis,” No. Pro00120887, August 29, 2022.

DEDICATION

I would like to dedicate this thesis to my wife, Katie, and my children Elliott, Gavin, Ari, and Paxton. Your daily support and encouragement sustain me. A sincere expression of gratitude to the AAA team, Dr. Anjali Bhagirath, for all your friendship, guidance and help in the lab, and Dr. Anum Haider, for your friendship and great conversations. Thank you to Dr. Khaled Altabtbaei for your patience, teaching, mentorship, and kindness in lab, clinic, and life. Thank you to Dr. Monica Gibson for pushing us to greater success than we would have achieved on our own. And thank you to Dr. Deanna Williamson for the conversation and curiosity as we write our story.

ACKNOWLEDGEMENTS

Thank you to all those who have supported me and my research in completion of this Masters thesis project. Thank you to the families for your participation and interest in science. Thank you to Dr. Khaled Altabtbaei, for your tireless dedication, support, guidance, and help on this project. Thank you to Dr. Anjali Bhagirath for your help and friendship in the lab and in our perio journey. Thank you to Dr. Monica Gibson for always encouraging us and pushing us to achieve more. Thank you to my wife and children for your support, patience, and prayers. And thank you to all those who have supported the perio grad program at the University of Alberta for your dedication, support, and patience with each graduate student as we pursue our goals and aspirations.

TABLE OF CONTENTS

THESIS ABSTRACT ..... **II**

PREFACE ..... **IV**

DEDICATION ..... **V**

ACKNOWLEDGEMENTS ..... **VI**

TABLE OF CONTENTS ..... **VII**

LIST OF FIGURES ..... **IX**

LIST OF TABLES ..... **X**

LIST OF ABBREVIATIONS ..... **XI**

CHAPTER 1: INTRODUCTION **1**

CHAPTER 2: HYPOTHESIS AND OBJECTIVES **9**

CHAPTER 3: MATERIALS AND METHODS **10**

CHAPTER 4: RESULTS **15**

4.1 Oral niche is the main driver of microbial separation 15

4.2 Saliva is the conduit to oral cavity microbiota 16

4.3 Sources of the reestablishment of the subgingival oral microbiome 18

4.4. Demographics of the population 24

4.5 Close contact activities. Vehicles of transmission? 25

CHAPTER 5: DISCUSSION 28

CHAPTER 6: CONCLUSION AND FUTURE DIRECTIONS 33

6.1 Conclusion 33

6.2 Strengths and Contributions 34

6.3 Future directions 35

6.4 Limitations and Weaknesses 36

REFERENCES 38

APPENDIX 41

LIST OF FIGURES

Figure 1: Oral health determinants as illustrated in Wolf et al (Wolf et al., 2021). 3
Figure 2: The classical "Pathogenesis of periodontitis," as illustrated in Page and Kornman et al. (Page, 1997). 3
Figure 3: Schematics of the steps that were done for the study (original image produced by Anjali Bhagirath using Biorender.com) 11
Figure 4: Oasis Diagnostics products. A: Micro•SAL™ for Children. B. DNA•SAL™. 11
Figure 5: Principal Coordinates Analysis (PCoA) of Phylogenic Isometric Log Ratio (PhILR) dissimilarity matrix. 16
Figure 6: Total contribution to salivary microbiota from intraoral sources for all participants. 17
Figure 7: Total contribution of intraoral sources of the child to the subgingival microbiome of the child. 19
Figure 8: Total contribution to the subgingival plaque of the child, with other individuals as the source, based on relationship to the child. 20
Figure 9: Total contribution to the subgingival microbiome of the child by sample type from all family members. 21
Figure 10: Total contribution to the subgingival microbiome of the child, sibling contribution of all sample types only. 22
Figure 11: Distribution of caregiving for the youngest child. 25
Figure 12: Distribution of families with caregiving other than mother or father, location of where the care is provided. 25
Figure 13: Scatterplot of the frequency of activity (mother close sleeping with child (x-axis) and the contribution of the saliva (y-axis, as a proportion). 26
Figure 14: Scatterplot of the frequency of activity (father kissing the child's cheek (x-axis) and the contribution of the buccal mucosa (y-axis, proportion). 27

LIST OF TABLES

Table 1: Total contribution to salivary microbiota by source, all individuals. 17
Table 2: Contribution of intraoral sources of the child to the subgingival microbiome of the child. Subgingival microbiome of the child is the sink, all oral sources of the child is the source. 19
Table 3: Statistical analysis of number of siblings, age, and gender on sample contribution.....23
Table 4: Demographics of the participants. 24

LIST OF ABBREVIATIONS

ASV	Amplicon sequence variant
NCD	Noncommunicable disease
EFP	European Federation of Periodontology
WONCA	World Organization of Family Doctors
WHO	World Health Organization
GWAS	Genome-wide association study
A.a.	*Aggregatibacter actinomycetemcomitans*
P.g.	*Porphyromonas gingivalis*
OTUs	Operational taxonomic units
CPITN	Community periodontal index of treatment needs
HMP	Human Microbiome Project

Chapter 1: INTRODUCTION

Periodontitis. The Non-communicable Oral Disease.

The global burden of diseases study, which examined 333 health conditions and 84 risk factors, has classified conditions into three main categories: communicable diseases, noncommunicable diseases (NCDs), and injuries. Under communicable diseases, further classifications include diseases of infectious etiology, maternal and neonatal mortality (regardless of cause), and deaths due to nutritional deficiencies. NCDs are a broad category that encompasses a variety of conditions, such as cardiac conditions, diabetes, cancers, chronic pulmonary diseases, mental health conditions, and many others (Mohan et al., 2019). Although the difference between communicable and noncommunicable diseases is wholly semantic in nature, the key distinction lies in whether a condition is transmissible. For example, diabetes and obesity are not transmitted by infectious agents, therefore they are not communicable diseases despite their prevalence in certain communities and not others, and intergenerational transmission of diabetes and obesity (Allen & Feigl, 2017). Therefore, NCDs may be best described as chronic conditions that are not spread through infection, and are characterized by their long-lasting nature, multiple risk factors, long development time, prolonged duration, and the potential to cause lasting impairment or disability (Piovani et al., 2022).

The World Health Organization considers the two most common oral diseases, dental caries and periodontitis, as noncommunicable diseases (Organization, 2020). Dental caries is the loss of tooth hard tissues (enamel, dentine, and/or cementum) caused by the dental biofilm that contains bacteria that can ferment sugars to acids (Wolf et al., 2021). The disease is multifactorial and is modified by diet. Periodontal disease is a chronic inflammatory condition that affects the tooth-supporting tissues (the periodontium). It begins with a reversible phase known as gingivitis. In certain individuals with compromised immune systems ('susceptible hosts'), gingivitis can progress to periodontitis — an irreversible destruction of the periodontium (Wolf et al., 2021). In this manuscript, we will concentrate on periodontal diseases, understanding that many factors can contribute to both caries and periodontal diseases.

Wolf et al. (2021) proposed that oral health is determined by a variety of factors that span from global issues to personal choices taken by the disease sufferer (figure 1.1(Wolf et al., 2021). It is generally agreed upon that periodontitis is a best understood by the model proposed by Kornman and Page (Page & Kornman, 1997) (figure 1.2), which illustrates that the pathogenesis of disease is an interplay between oral microbial insults and the host immune system reacting to it, with genetic, environmental, and acquired factors modifying this interplay.

In recent years, the concept of periodontitis as a NCD has been endorsed by periodontist scientific and professional groups. In 2023, the Joint Workshop by the European Federation of Periodontology (EFP) and the European arm of the World Organization of Family Doctors (WONCA Europe) published a consensus that grouped periodontitis with other noncommunicable diseases (Herrera et al., 2023). The consensus group also recommends that dentists and physicians collaborate in managing NCDs, with the EFP clinical practice guidelines published after the World Workshop in 2017 being central in managing periodontal disease. The clinical practice guidelines recommend dentists develop personalized care plans to effectively treat periodontal conditions (Herrera et al., 2023) This personalized approach puts the patient at the center of intervention, with the therapy being directed toward them, and the continued success of this therapy is determined by the actions of the individual with disease. Therefore, the aforementioned oral health determinants by Wolf et al. (figure 1) are still very much in line with the classical Page and Kornman pathogenesis of periodontitis (figure 2), which also puts the patient at the center, with many of these health determinants being grouped into the “environmental and acquired risk factors” in that model.

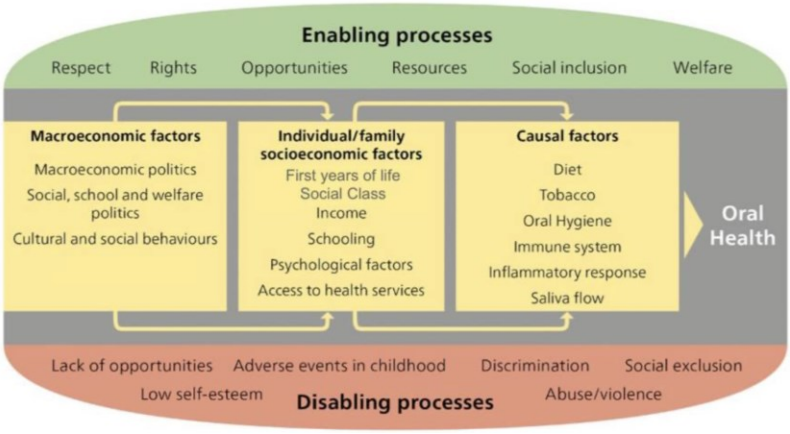


Figure 1: Oral health determinants as illustrated in Wolf et al (Wolf et al., 2021).

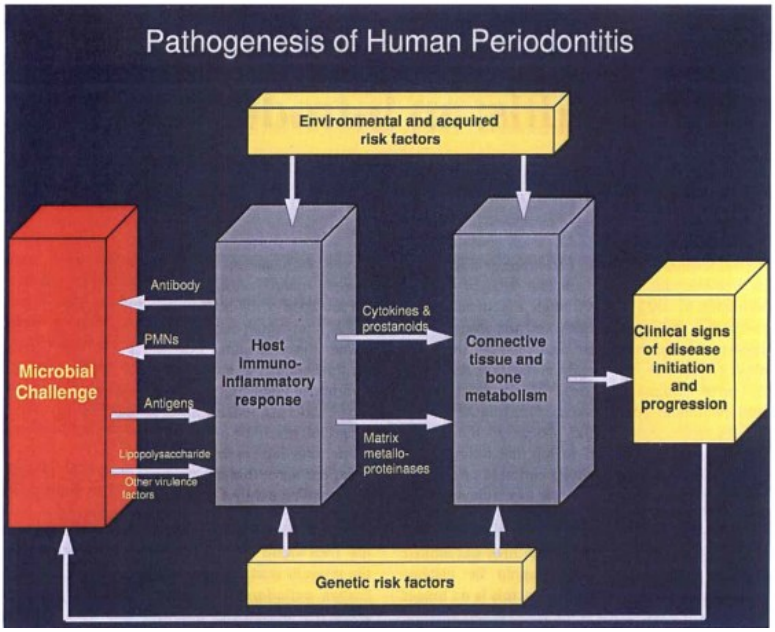


Figure 2: The classical "Pathogenesis of periodontitis," as illustrated in Page and Kornman et al. (Page, 1997).

Non-communicable ≠ Non-transmissible

Periodontal disease stands out among WHO-classified conditions due to a diverse microbiota being its primary causative agent. This causation by multiple species greatly complicates the understanding of its pathogenesis. Unlike typical communicable diseases, which generally follow a 'one bug, one disease' model, periodontal diseases involve approximately 1,000 different microorganisms (Berger et al., 2018). Many of these microorganisms reside in the subgingival

pocket, where their accumulation and activity contribute to the disease. It is well documented that microbiota is transmissible, and that the microbial influence in health and disease should not be ignored (Alanazi, 2022; Asikainen, 1999; Brito, 2019; Könönen, 2000; Monteiro, 2021; Reis, 2023; Tuite-McDonnell, 1997; Valles-Colomer, 2023; Van Winkelhoff, 2005; von Troil-Lindén, 1997; Von Troil-Lindén, 1995). However, much evidence in literature conflicts with each other, and we believe one major contributor to this is the reliance on models which fail to acknowledge the effect of the microbiota. Although the following is not an exhaustive review of the literature on how the microbiota can influence these models, we at least hope to illustrate that the transmissibility of micro-organisms in the oral cavity is a non-trivial issue that should not be ignored.

Heritability studies explore the extent to which genetic differences among individuals within a population contribute to variations in a trait. Nibali et al. (2019) estimated that the heritability of periodontitis is 38% in twin studies but drops to just 7% in Genome-Wide Association Studies (GWAS) (Nibali et al., 2019). In twin studies, traits in monozygotic (identical) twins—who share almost all their genes—and dizygotic (fraternal) twins—who share about 50% of their genes—are compared using the Falconer equation. Ideally, if a trait is purely genetic, it would manifest twice as frequently in monozygotic as in dizygotic twins. Deviations from this pattern suggest environmental influences. This "environment" includes everything immediately external to the individual's body. The host's microbiome, which does reside on the body of the individual, is misattributed to the genetics of the person (Awany & Chimusa, 2020). Without accounting for this, the models produce erroneous results. For instance, twin studies have shown that tooth brushing frequency, spitting and rinsing after brushing, length of cohabitation and romantic relationships reduce the genetic fraction (M. Freire et al., 2020; Demmitt et al., 2017). This is counterintuitive as these habits do not alter the genetic makeup of the twins; they simply influence the microbiome on the host that the twin models falsely attribute to the host's genetics.

Another method of studying disease is by examining families. The heritability of periodontitis is estimated to be 15% in family studies (Nibali et al., 2019). It has been observed that children of parents diagnosed with periodontitis are more likely to develop periodontitis themselves (Alanazi, jumah Alturaif, et al., 2022). These family studies examine disease based on penetrance, or how

often a disease is present in the background of inherited collection of specific genes. Unfortunately, these studies do not account for microbial transmission.

Other familial studies have explored this microbial transmission, using both microbe-specific and open-ended metataxonomic approaches. The number of studies that have examined these questions in detail are scarce, and the high-level of evidence studies will now be reviewed. Microbial transmission is not uniform for all bacterial species. For example, *Aggregatibacter actinomycetemcomitans* (*A.a.)* is rarely found in cohabitating adults but is frequently detected between children and their parents (Asikainen & Chen, 1999). On the other hand, transmission of *Porphyromonas gingivalis* (*P.g.)* follows a different transmission route, as the presence of one *P.g*-positive individual in the family increases the likelihood of detecting *P.g* in other family members (Asikainen & Chen, 1999). Vertical transmission (from parent to child) is estimated to be 30-60% for *A.a*, whereas it is seldom seen for *P.g*. On the other hand, horizontal transmission rates show that *P.g.* is transmitted between spouses at a rate of 30-75%, compared to 14-60% for *A.a* (Van Winkelhoff & Boutaga, 2005). If the horizontal transmission of these periodontal pathogens is persistent, we expect to see their effect on the periodontium. Indeed, studies have shown that periodontitis in one spouse often leads to poorer periodontal conditions in the other, potentially through the transmission of oral bacteria (von Troil-Lindén et al., 1997). Additionally, a longitudinal study that attempted to disrupt the biofilm in adults with periodontitis could not fully rule out an exogenous source contributing to the biofilm (von Troil-Lindén et al., 1996). Furthermore, a series of case-control studies from Brazil found that families share more similar microbial compositions in saliva (Monteiro et al., 2014), subgingival plaque (M. F. Monteiro et al., 2021), and the microbiome response to the subgingival microbiome (Monteiro et al., 2022), with these similarities being dependent on age (Reis et al., 2023). Remarkably, this microbial similarity and their deleterious effect persists even though children of parents with periodontitis maintain oral hygiene levels comparable to those children from families without a history of the disease. This is important because although the acquisition of an oral microbiome is a dynamic process which starts early in a child's life, the most important acquisition is arguably the one that occurs during the period when the child is shedding their primary dentition and the beginning of the eruption of the permanent dentition, leading to what is known as the mixed dentition state. The acquired microbiome at this stage is relatively resilient to change (Reis et al., 2023), and various taxa persist

throughout life in a stable manner, forming a part of the core of the adult microbiome (Mason et al., 2018).

When Everything is Everywhere, What Environment Selects?

As Becking famously stated (de Wit & Bouvier, 2006): "Everything is everywhere, but the environment selects." This concept is crucial in understanding microbial genetic exchanges, which studies have shown to be more frequent in smaller, localized environments than in larger ecosystems (Brito et al., 2019). Such findings are pivotal when considering a global disease like periodontitis. By understanding the specific environmental factors that influence the acquisition of perio-pathogens the most, we can better target our efforts to best curb their spread and impact. This approach requires a detailed understanding of the 'environment' – ranging from human tissues to global ecosystems – and the complex networks of variables involved as these environments are not discrete but overlapping, forming a continuum where microbiomes can move between environments. This makes isolating the effect of one environment from another very difficult. Moreover, periodontal diseases are caused by the subgingival plaque, which is difficult to sample without contamination, making its use in large-scale studies difficult. Fortunately, salivary research has emerged as an attractive alternative research tool as it carries the microbiota of several oral niches such as tongue, cheek, supra- and sub-gingival plaque, and it is easily accessible, making it an ideal method in population surveys (Demmitt et al., 2017). In the next two sections, we will summarize two high quality studies which illustrated environmental transmission between various environments, while minimizing external microbiota transmission between environments.

In a relatively isolated cohort of 287 people in the Fiji islands, strong transmission patterns were seen within households especially between spouses (Brito et al., 2019). Cohabiting individuals showed a median oral strain-sharing rate of 32%, which is significantly higher than the 3% in non-cohabitating individuals in the same population, and the 0% in non-cohabitating individuals in different villages. Additionally, similar microbiota were found in mother-child pairs and within spousal pairs (Valles-Colomer et al., 2023). The similarity in the microbiota in mother-child pairs and between spouses suggests person-to-person transmission or a shared environmental source, which is especially pertinent as these spouses were not genetically related. Notably, the duration of cohabitation was positively associated with strain sharing more significantly than the age or

genetic relatedness of the individuals (Valles-Colomer et al., 2023). Families with high levels of microbial transmission experienced higher transmission across all kin relationships regardless of genetic relatedness of the individuals, likely due to more close contact physical interactions with each other. This pattern of close-contact mediated microbial sharing was evident even when they only started in adulthood (e.g., 38% of oral strain sharing between partners). Interestingly, this strain sharing was partially reversible over time; twins who lived apart for 30 years showed a decrease in strain sharing from 30% to about 10% (Valles-Colomer et al., 2023). The same patterns were seen when compared to people in the United States (Valles-Colomer et al., 2023). Gram-negative species were most highly transmitted in households, compared to mother-child transmission, and intra-population sharing (Valles-Colomer et al., 2023). Taken together, this illustrates that families are the most critical environment for salivary microbial transmission.

A Mars-travel simulation study provided a good opportunity to examine salivary microbiota dynamics under a highly controlled environment (Bacci et al., 2021). The six astronauts were isolated in a Mars simulation pod with no outside contact for approximately a year and a half, which meant that the only microbial exposure that these astronauts had came from either the highly controlled environment around them, or from the astronauts themselves. Once the astronauts began the study, that is, once isolation began, the diversity of the salivary microbiota decreased. However, the differences between subjects remained relatively unaltered throughout the study, suggesting that individuals follow relatively independent dynamics in their salivary microbiota. Changes seen within each crew member's saliva followed a unique and personal trajectory. The microbiota that remained stable, which was shared by most crew members and was detectable at most time points, consisted of 144 stable amplicon sequence variants (ASVs) compared to 1746 unstable ones. This stable microbiota also exhibited a higher abundance compared to its unstable counterparts. These findings highlight that the salivary microbiota is relatively stable over time, but is also a dynamic entity, influenced significantly by environmental conditions, where most of the microbial members in saliva were of low abundance and were transient in nature.

In summary, while saliva serves as a conduit for microbiota transfer among individuals, using it to assess oral microbial acquisition is not without challenges. Nonetheless, it clearly demonstrates the dynamic interaction between environmental conditions and microbial transfer, particularly

highlighting that close interpersonal contact, especially within households, is a primary means for spreading microbiota.

For microbiota to transfer from one individual to another and impact periodontal health, it must be transmitted via the donor's saliva, survive the journey to the recipient, and ultimately establish itself within the recipient's subgingival plaque. Unfortunately, comprehensive evidence supporting such transmission remains scarce. Additionally, there is a lack of data on the contribution of non-parent household members to the subgingival microbiome. Furthermore, there are no studies that quantitatively assessed the contribution of non-self-individuals to the subgingival plaque, particularly when it comes to what oral sources participated in this subgingival acquisition.

In this study, we explored alterations in the oral microbiome at an age where children are susceptible to oral microbiome changes (Könönen, 2000). We conducted a thorough oral prophylaxis to induce an “environmental collapse” of the oral microbiome. After this intervention, we monitored the recolonization process, aiming to identify the sources of bacteria that have recolonized the subgingival plaque. This included sampling various oral sites such as saliva, tongue, buccal mucosa, supragingival plaque, and subgingival plaque. We attempted to concentrate on preschool children who predominantly spend time at home to minimize external microbial influences. Sampling was conducted on entire family units, including multiple children per household, to facilitate the study of microbial transmission among non-parent family members.

Chapter 2: HYPOTHESIS AND OBJECTIVES

Rationale: Gingivitis and periodontitis are defined as noncommunicable, microbially mediated diseases. Previous studies have shown that this subgingival microbiome is shared across different family members. Studies that examined the spread of salivary microbiome in a variety of environments (cities, towns, families, filial and non-filial relationships) have demonstrated that the largest sharing occurs in close contact, with households being the largest contributor to this sharing. Microbial diseases are generally treated with the individual in mind; however, such therapies have generally not provided lasting impact on the subgingival microbiome. Despite therapeutic treatment, the relative stability of microbiota along with strong household transmission leads to the priming of disease-naïve children to future incidence of the same periodontal disease that their parent(s) have. Therefore, to understand the familial environment and its effect on the acquisition of subgingival microbiome, we must study this acquisition in a longitudinal fashion through the re-establishment of the subgingival biofilm microbiota after its removal, while tracking the sources that contributed to it. Put more formally, we hypothesize that the subgingival biofilm is re-established from sources both inside the oral cavity of the person, and outside of the person themselves.

The study design was focused on three specific aims:

Aim 1: Identify the sources of subgingival microbiome within an individual.

Aim 2: Identify the effect of intimate contact between parents and child as well as between siblings and child in vertical and horizontal transmission of the microbiome.

Aim 3: Examine the effect of recolonization on the child after professional oral prophylaxis.

Chapter 3: MATERIALS AND METHODS

Study structure: Ethical approval was obtained for this study (study number: Pro00120887). Fourteen families were recruited for this cross-sectional pilot study. Inclusion criteria for families required that families had at least one parent in the home, one child of preschool age (≤5 years old, primary, or mixed dentition phase), and at least one sibling. Families were recruited from local churches and other community organizations.

Informed consent to participate in the study was obtained from all participants. Participants were brought to the Oral Health Clinic of the University of Alberta, and samples were collected from all family members as outlined in figure 3 and below.

Visit 1: A sample of the saliva was collected from the parents, child, and other family members. Next, the parents, child, and siblings had their other oral niches sampled (buccal mucosa, tongue, supra- and sub-gingival plaque) using the Oasis Diagnostics (Vancouver, Washington, USA) products as shown in figure 4. Briefly, product A was used for saliva collection from child subjects, and product B was used for soft tissue swabs of the buccal mucosa and the dorsum of the tongue. Sterile tubes were used to collect saliva from adult participants. Sterile paper points were used for collection of supragingival plaque taken at several intraoral sites then pooled together in one tube. Subgingival plaque samples were likewise collected with sterile paper points placed subgingivally into the sulcus at the distal of the 32, 31, 42, and 41 for 20 seconds at each site. Sterile paper points were then placed in 40uL of RNA*later*. All intraoral samples were then placed in a -20^{o}C freezer.

Dental prophylaxis for the subject of study, i.e. the preschool aged child, was then performed. This served as the "ecological catastrophe" that effectively disrupted the biofilm, allowing for a reset of the subgingival microbial community.

Visit 2: A saliva sample was collected, as well as the other oral niches sampled from the pre-school aged child (the subject). These included the buccal mucosa, tongue, supragingival plaque,

and subgingival plaque. All collected samples were stored in -20C freezer until time for DNA isolation.

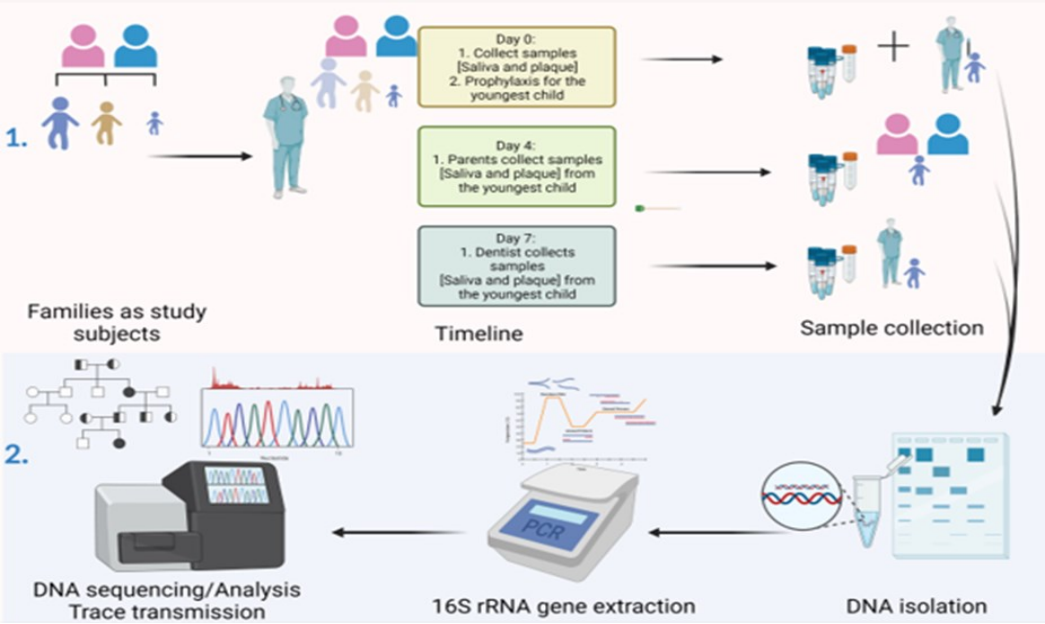


Figure 3: Schematics of the steps that were done for the study (original image produced by Anjali Bhagirath using Biorender.com)

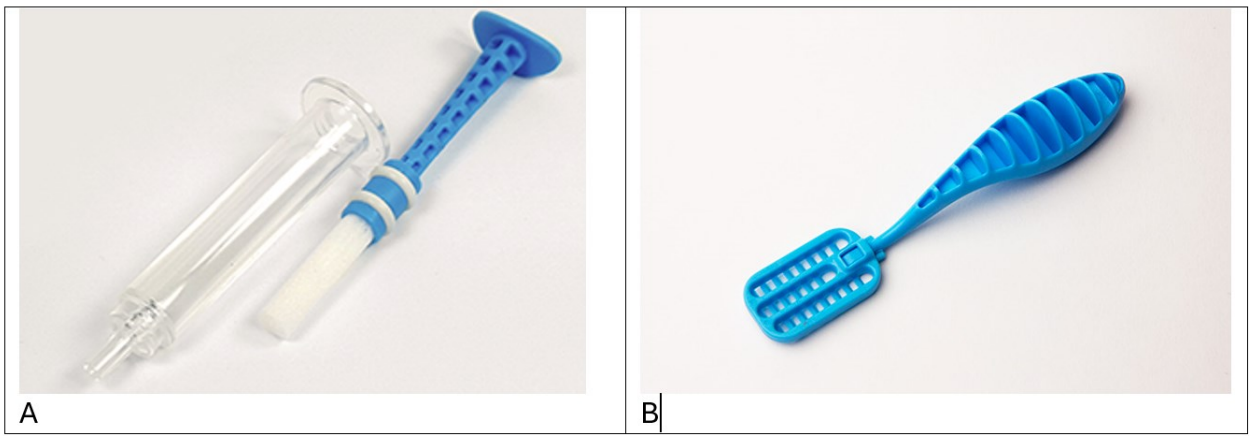


Figure 4: Oasis Diagnostics products. A: Micro•SAL™ for Children. B. DNA•SAL™.

Photos of products from the seller's website (https://4saliva.com/products/)

Close contact questionnaire: A structured questionnaire (appendix A) was developed and administered to the mothers/fathers. The questionnaire gathered information on the family demographics, socioeconomic status, education status, general health of the family members, and close contact activities within the family. The questionnaire asked about intimate contact that they and other family members (including grandparents, caregivers, etc.) had with their child as part of caregiving and play activities with the goal of identifying which activities significantly affected the transmission. For example: it asked to approximate the time that parents, caregivers, and sibling spent with the child, which member in the family/caregiver was responsible for different aspects of childcare such as feeding and playing, and whether the child sleeps with another individual in the household (and as such breathes aerosols from that individual. The structured questionnaire also included questions about contact that requires minimal exposure to air including kissing a child's lips, sharing eating and drinking utensils, pre-chewing food for infants, sharing the same toothbrush, and cleaning the pacifier orally.

Laboratory analysis: Phosphate buffered saline was added to the tongue scraper, and paper points, and vortexed for 1 hour before leaving them to settle. Saliva was processed as is. All samples were processed by QIAGEN QIAamp DNA Mini Kit (Germantown, MD, USA) according to manufacturer's instructions. Genomic DNA concentration was quantified by fluorometric analysis using the Qubit® 4 Fluorometer system (Invitrogen by ThermoFisher Scientific - Q33238) with the Qubit™ 1x dsDNA High-Sensitivity Assay Kit (Invitrogen by ThermoFisher Scientific – Q33231).

Amplicon sequencing was done in Genome Quebec core facility to amplify the V1-3 region using the 27F/519R primer, with the following sequences:
Forward primer 5'-
ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAKRGTTYGATYNTGGCTCAG-'3,
and reverse primer 5'-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGTNTBACCGCDGCTGCTG-'3.

Computational analysis: Sample pre-processing was done 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. The preprocessing steps are detailed below. Primers were trimmed using cutadapt (Martin, 2011). Parameters for denoising with DADA2 (Callahan et al., 2016) were determined with the following steps.

1) Sequences were copied to working files and inputted using SeqKit (Shen et al., 2016). The lengths were measured and those shorter than 25th percentile length were discarded (Shen et al., 2016).

2) Next, SeqKit trimmed all sequences from their '3 end to match the length of the 25th percentile.

3) The trimmed sequences were then used in FIGARO (Weinstein et al., 2019) to determine the input parameters for DADA2.

The denoising process with DADA2 is as follows:

1) Sequences are first fed to the learning errors algorithm to construct the denoising model.

2) Next, sequences are denoised using the generated denoising model.

3) Chimeric sequences (those that are generated due to sequencing errors that join 2 unrelated sequences into one) are removed after generating a model of all abundant sequences and comparing the sequences to that model.

4) Next, denoised sequences that are identical to each other but only vary in their '5 or their '3 due to longer sequencing are collapsed into one sequence.

At this point, the denoised sequences complete the denoising process and are considered as amplicon sequence variants (ASVs). These ASVs are 100% homologous to what was present in the microbiota with no sequencing errors. This greatly enhances the resolution of sequencing than the previously used 99% similarity cut-off which produces operational taxonomic units (OTUs). This is because even when extremely similar but non-identical sequences are found, 99% similarity

would condense the two into the same OTU, while ASVs will not condense them into a single sequence, which greatly enhances our resolution without blurring the line between samples. This is useful in situations where, for example, if the sequences of OTU-A and OTU-B are extremely similar but only differ in a single nucleotide, 99% similarity would have assigned the same OTU to both sequences. Therefore, if OTU-A is only present in the parent, but the child has both OTU-A and OTU-B, it is not possible to identify that the child has two OTUs with possibly two sources. The enhanced clarity provided by ASVs mitigates this shortcoming.

ASVs were then fed into a Bayesian analysis model (SourceTracker2 (Knights et al., 2011)), which estimates the contribution of various samples (called sources) into a target sample (called sink). A model was developed where the subgingival sample of the child 1 week after the prophylaxis was identified as a sink, and all the samples from the child and all family members were identified as the sources. The algorithm was run using the package's default values. Alpha and beta diversity (PhILR (Silverman et al., 2017)) metrics were measured and plotted using FALAPhyl, an open-source fully automated pipeline from the lab (GitHub.com/khalidtab/falaphyl).

Based on the results of the questionnaire, we examined all close contact activities through correlations to the sources that contributed to the child's subgingival bacteria. Spearman correlation test analysis was performed to determine correlation between close contact activities and sample contributions to the child subgingival plaque as the sink.

Chapter 4: RESULTS

To more fully understand familial contributions to the subgingival microbiome of the youngest child, we examined a number of broad categories. First, what is the main driver of microbial selection in each sample? Second, as saliva is the vehicle for transmission of oral microbes between individuals, the sources of the salivary microbiome were further investigated. Following perturbation of the child's microbiome, the sources of the recolonization of the subgingival microbiome were explored, both internal and external to the child. Finally, the correlation between close contact activities and transmission of the oral microbiome was examined.

4.1 Oral niche is the main driver of microbial separation

We started with examining the global drivers of separation between the oral samples. We examined univariate differences of oral niches (saliva, buccal mucosa, tongue, Supra- and sub-gingival; plaque), patient type (child, sibling, mother, father), and between family differences. The largest separation was seen in oral niches ($p<0.05$, ADONIS on PhILR distances – Figure 5a), which is independent of the individual's designation (Figure 5b) and independent of the family allocation (Figure 5c). In other words, significant similarity can be seen in the same samples from different individuals. This similarity is higher than the similarity between samples from different niches from the same individual and from individuals from the same or different families. Therefore, the microbiota is mainly driven by sample types, which is indicative that across different individuals, similar environmental pressures exist that are highly selective of the microbiota capable of occupying it, tissue interface (hard or soft tissue; shedding or non-shedding), availability of nutrients, aerobic/anaerobic status, and interaction with the host immune response. Any other effect is secondary to the sample type.

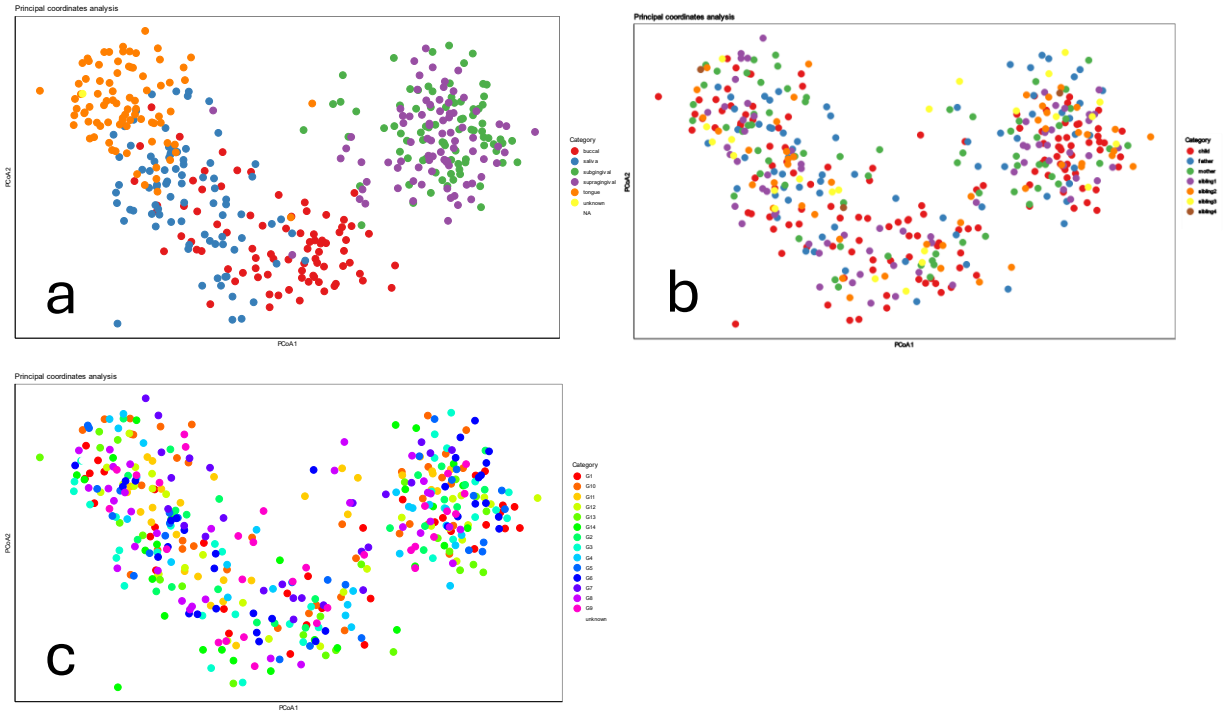


Figure 5: Principal Coordinates Analysis (PCoA) of Phylogenic Isometric Log Ratio (PhILR) dissimilarity matrix.

Each dot represents a sample. The three plots are identical and only differ in the colors of the dots, which represent the univariate variable categories. Figure 5a: graph plotted to emphasize intraoral niche, 5b: graph plotted to emphasize parent, child and sibling status. Figure 5c: graph plotted to emphasize familial allocation.

4.2 Saliva is the conduit to oral cavity microbiota

Saliva is the main vehicle of the transmission of the oral microbiota between individuals. To understand the sources of the microbiota in saliva, we ran a Bayesian source tracking model with saliva as the sink, and the following oral niches as sources: subgingival plaque, supragingival plaque, buccal mucosa, and tongue. Results are shown in figure 6 and table 1. The tongue contributed the most microbiota (median=58.9%), followed by buccal mucosa (median=14.8%), supragingival plaque (median=1.1%), and finally subgingival plaque (median=0.8%). Untraced/unknown sources contributed 12.5%, which could be either environmental species, or

microbiota inherent to saliva itself. As can be seen, the subgingival plaque as the source contributed the smallest percentage of the total salivary bacterial population (median=0.8%).

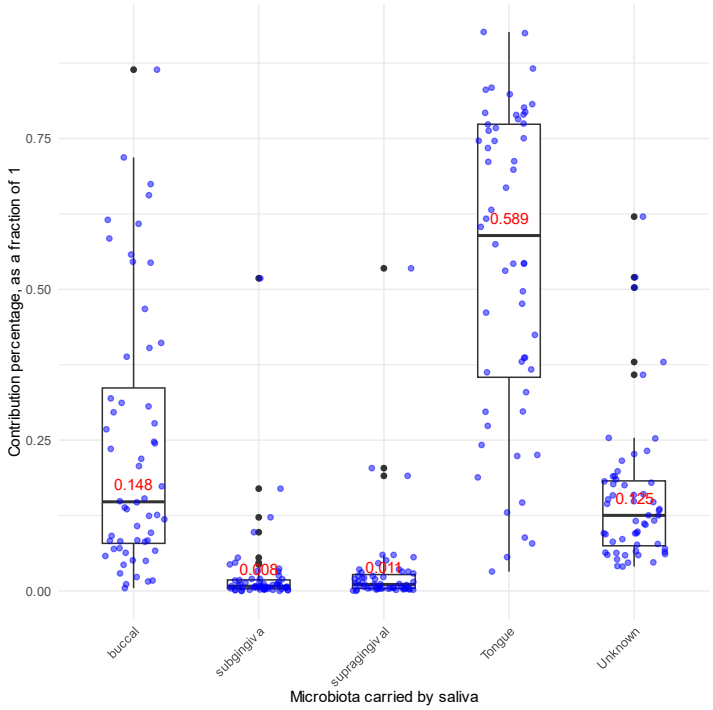


Figure 6: Total contribution to salivary microbiota from intraoral sources for all participants.

Values given are a fraction of 1, with the line inside the box as the median, and the extensions of the box as 25th and 75th percentile. The whiskers from the box are the interquartile range. Dots represent data points.

Table 1: Total contribution to salivary microbiota by source, all individuals.

Source	Total contribution (median %)
Tongue	58.9
Buccal mucosa	14.8
Supragingival	1.1
Subgingival	0.8

Unknown	12.5

4.3 Sources of the reestablishment of the subgingival oral microbiome

To understand the sources contributing to the re-establishment of subgingival plaque, we constructed a Bayesian source tracking model where we designated the oral samples of the child and the family members as sources, and the subgingival plaque of the child 1 week later as the destination. The algorithm's nomenclature refers to the subgingival plaque as the sink. This terminology will be used in the rest of this thesis. This model specifically examines whether there is a contribution from external oral sources to the child's subgingival plaque.

We considered the intra-child samples first (buccal, tongue, saliva, supragingival, and subgingival). These results are summarized in figure 7 and table 2. For the intra-child sources, 46% of the subgingival plaque at 1 week was traced to the previous subgingival plaque (from baseline, time 0), 9% from supragingival, 1% from buccal, and negligible amounts from saliva and tongue. That is, for the intra-child sources, the main contributor to the subgingival plaque at 1 week was the subgingival plaque at time 0, followed by supragingival plaque, followed by buccal mucosa.

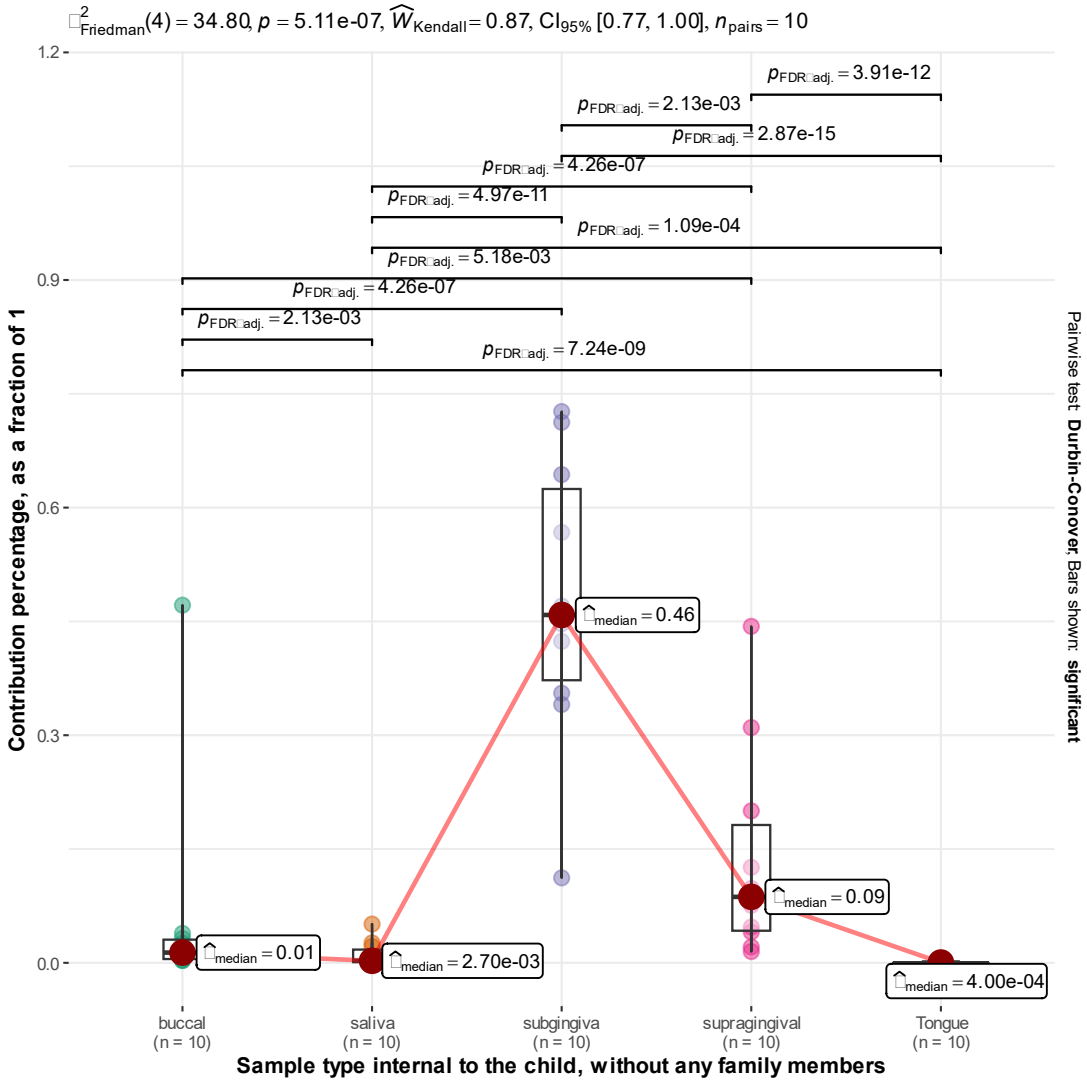


Figure 7: Total contribution of intraoral sources of the child to the subgingival microbiome of the child.

Subgingival microbiome of the child at one week is the sink, all samples of the child at time 0 are the source.

Table 2: Contribution of intraoral sources of the child to the subgingival microbiome of the child. Subgingival microbiome of the child is the sink, all oral sources of the child is the source.

Source	Contribution (median %)
Subgingival plaque	46%

Supragingival plaque	9%
Buccal mucosa	1%
Saliva	Negligible
Tongue	Negligible

Next, we focused on all contributions, including from family members. When considering the contribution of the individual family members to the repopulation of the oral microbiome in the child, 65% of the subgingival microbiome came from the child, 8% came from the siblings, and 3% came from mother and father, respectively (fig. 8). This suggests that the role of the siblings' microbiome is a more significant contributor to the oral microbiome of the child than either parent. It also suggests that the contribution from mother or father is not statistically different.

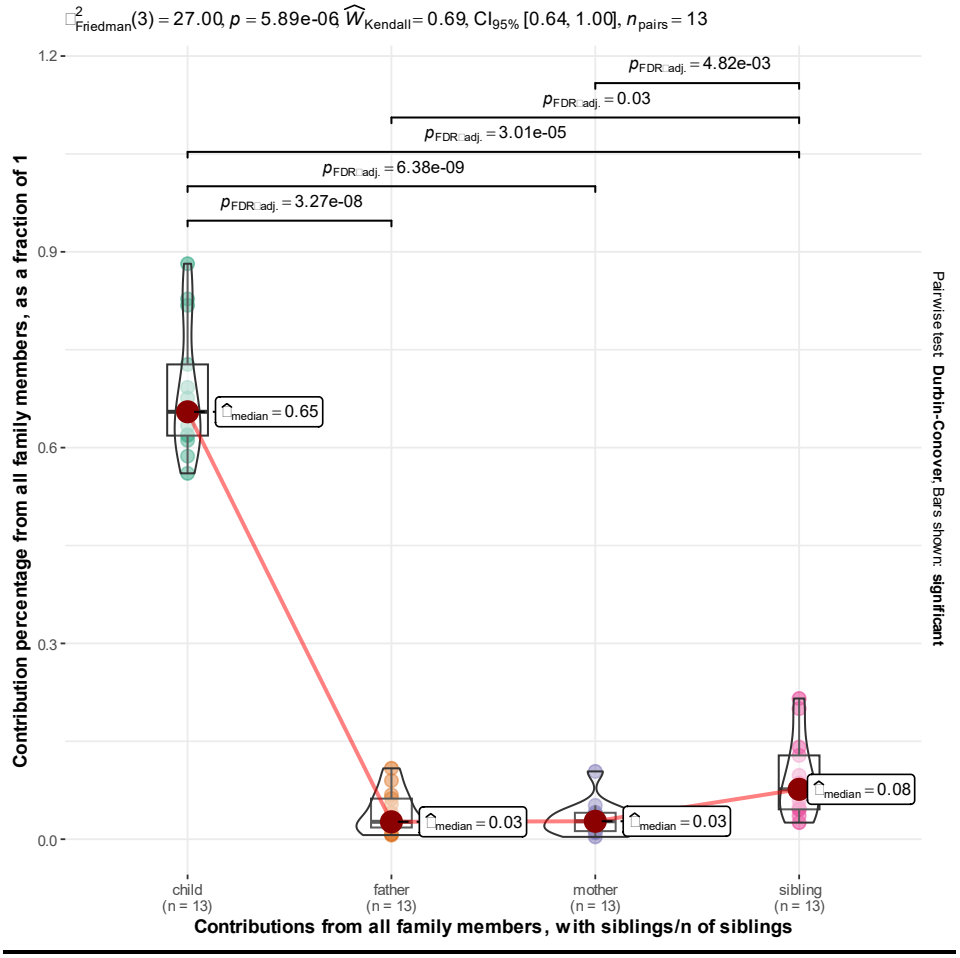


Figure 8: Total contribution to the subgingival plaque of the child, with other individuals as the source, based on relationship to the child.

Next, we looked at the contribution of different sample types from all family members to the re-establishment of the subgingival microbiota of the child (fig. 9). Surprisingly, the highest contribution from family members to the child was from the subgingival plaque (8%), followed by supragingival plaque (4%), followed by the buccal mucosa (1%). Contribution from the saliva and tongue of family members was negligible. That is, despite the fact that only a median of 0.8% of the microbiota in saliva came from subgingival plaque (fig. 6), approximately 8% of the sources external to the child came from subgingival plaque of family members (fig. 9). This is possibly due to the similarity of subgingival plaque composition across different individuals (figure 4.1), making it efficient in establishing itself into a new person.

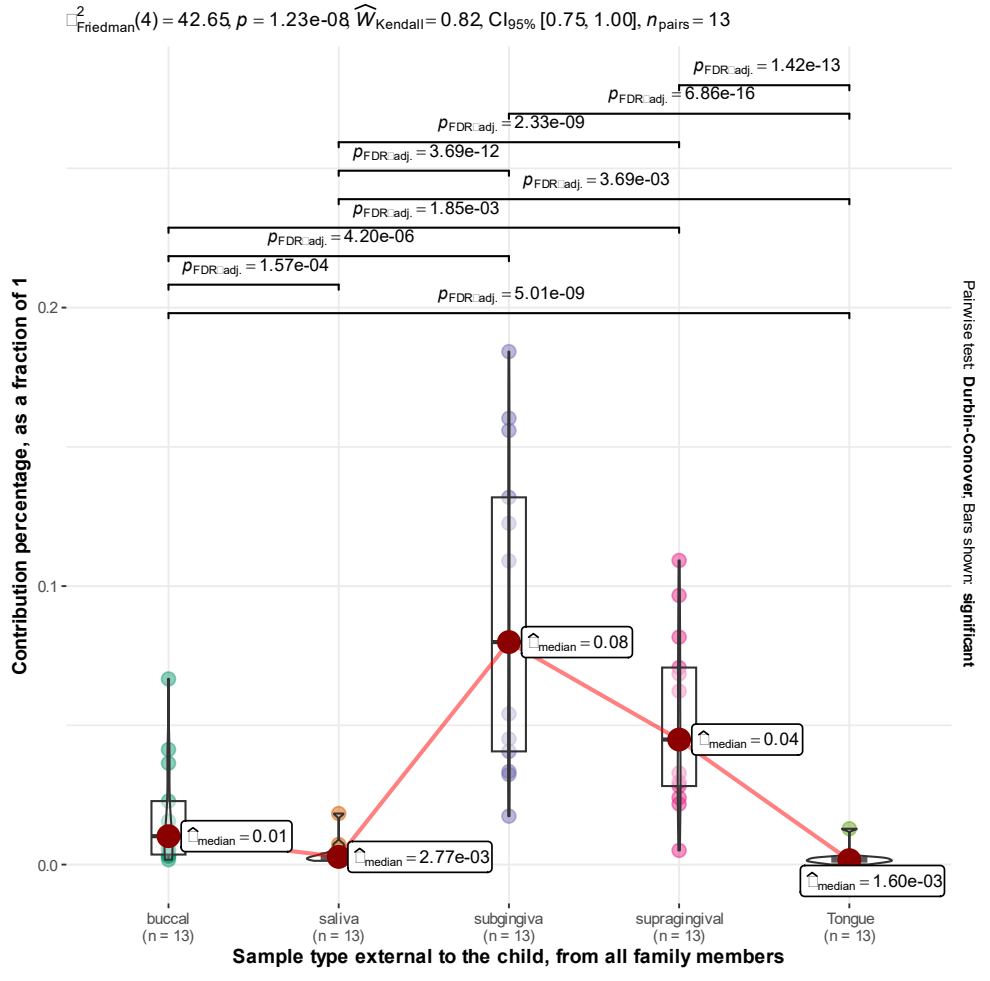


Figure 9: Total contribution to the subgingival microbiome of the child by sample type from all family members.

We also found similar results when we examined the contributions of different sample types from siblings only (fig. 10). The greatest contribution was from subgingival plaque (3%), supragingival plaque (2%), and negligible contributions for saliva, buccal, and tongue.

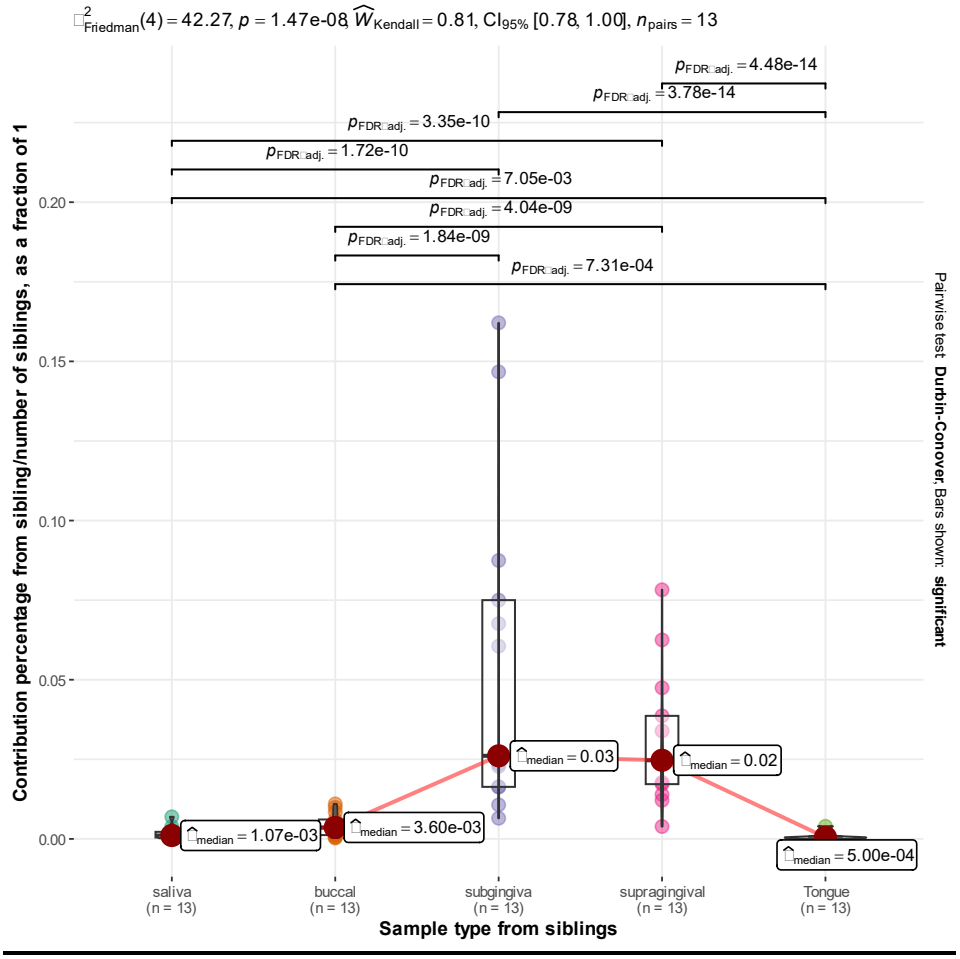


Figure 10: Total contribution to the subgingival microbiome of the child, sibling contribution of all sample types only.

Next, we wanted to know if the number of siblings had any influence on the level of contribution. A linear mixed effects model was formulated as below:

- lmer(log(contribution)~ HowManySiblings + (1|sampleType) + (1|family) -1, data=siblings)

where the family and the sample types were constructed as random effects. This is because we were interested in whether the number of siblings influences the percentage of contribution, regardless of sample type. Indeed, the number of siblings was statistically associated with the contribution in the child's subgingival plaque (Estimate=0.5094, Standard error=0.1381, df= 59.2199, t-value=3.688, p-value = 0.000493).

Next, we wanted to know if the age difference between the child and their sibling(s), sample type, and the sex of the sibling(s) (and their interaction terms) had any effect on the contribution, with family as a random effect. Subgingival plaque was used as the reference point for the intercept.

- lmer(log(contribution) ~ (sex * sampleType * ageDiff) + (1|family), data = siblings)

Age difference was not statistically significant. Next, we replaced it with whether the sex of the child and their sibling was the same or different, and that term was also not statistically significant. Therefore, both sex difference and age difference were dropped from analysis, and the model was rerun as:

- lmer(log(contribution) ~ (sex * sample type) + (1|family), data = siblings)

where sex is the male/female of the sibling, regardless of the sex of the child in question.

Table 3: Statistical analysis of number of siblings, age, and gender on sample contribution.

Category		Estimate	Std. Error	df	t-value	Pr (>|t|)
Intercept	Subgingival plaque	-3.5609	0.2771	80.5234	-12.852	<2e-16
Sample type	Buccal mucosa	-2.3136	0.3705	102.5834	-6.245	**9.73e-09**
	Saliva	-2.9358	0.3705	102.5947	-7.924	**2.90e-12**
	Supragingival plaque	-0.6905	0.3705	102.6145	-1.864	0.0652
	Tongue	-3.7306	0.3771	103.1259	-9.892	**<2e-16**
Sex	Female	0.2564	0.4728	109.8863	0.542	0.5887

Interaction terms	Female	Buccal mucosa	-1.3816	0.6480	102.4555	-2.132	**0.0354**
	Female	Saliva	-0.8549	0.6362	102.7764	-1.344	0.1820
	Female	Supragingival plaque	0.2130	0.6361	102.7031	0.335	0.7385
	Female	Tongue	-0.3852	0.6689	103.7162	-0.576	0.5659

When the sibling's sex was female, the contribution from buccal mucosa was statistically significantly lower than when the sibling's sex was male, compared to the levels of subgingival plaque between males and females.

4.4. Demographics of the population

In total, 14 families were recruited, with each family composed of one mother, one father, and at least two children. The youngest child was of pre-school age and was not yet enrolled in an educational institution. In total, 28 parents and 43 children participated.

Table 4: Demographics of the participants.

Participant	Number of participants
Mother	14
Father	14
Child	Male: 23 (53.5%)
	Female: 20 (46.5%)

As we wanted to examine the sources of contribution to the re-establishment of the subgingival microbiota, and the effect of close contact activities on the transmission of oral microbiota, we explored what individuals were responsible for the primary care-giving the children. Nine of 14 families had only mother and father care of the children once a week or more, 2 families had grandparents watching the children once a week or more, 2 families had daycare, and 1 family had a nanny.

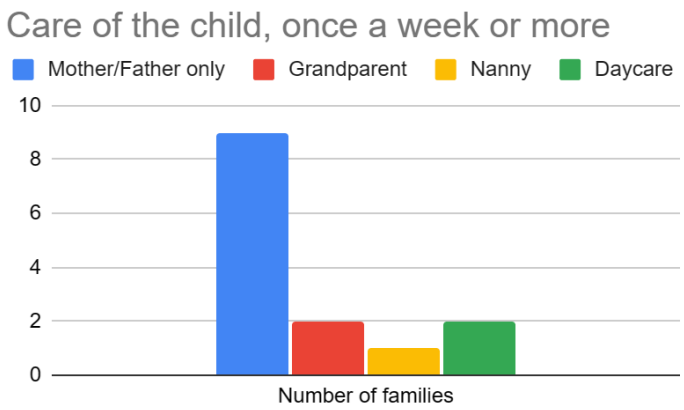


Figure 11: Distribution of caregiving for the youngest child.

Of 5 families with individuals other than mother or father taking care of the children once a week or more, 3 families had this care provided for in the home, and 2 families had the children in a daycare or day home (fig. 12)

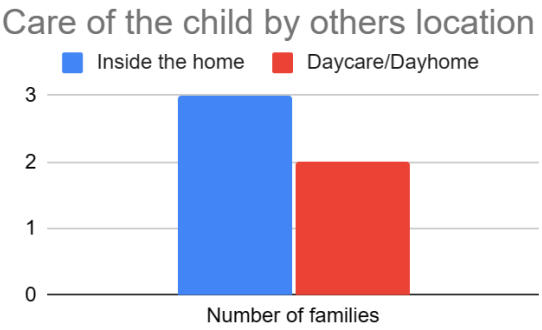


Figure 12: Distribution of families with caregiving other than mother or father, location of where the care is provided.

4.5 Close contact activities. Vehicles of transmission?

Understanding that there is transmission of saliva between individuals within the family, and that saliva acts as the vehicle for the transmission of other oral bacteria, we investigated close contact activities and their correlation with transmission of oral bacteria. Based on the results of the questionnaire, we examined all close contact activities through correlations to the sources that contributed to the child's subgingival bacteria. Based on Spearman correlation test analysis, three

close contact activities were seen to have a statistically significant correlation with contributions from family members to the child: close sleeping with the child by the mother (saliva) (Rho=0.641, p-value=0.0247), cuddling with the child by the mother (subgingival) (Rho=0.707, p-value=0.0101), and kissing on the child's cheek by the father (buccal) (Rho= 0.714, p-value=0.009). All other close contact activities did not make a statistically significant contribution to any of the samples of the child. When plotted on a scatterplot, only the correlation between mother close sleeping with the child and saliva contribution (see fig. 13), and father kissing the child on the cheek and buccal mucosa contribution showed a meaningful trend-line (see fig. 14). This is because mother cuddling with the child and the subgingival contribution was not meaningful as all participants except 1 answered the same frequency of this activity (see appendix).

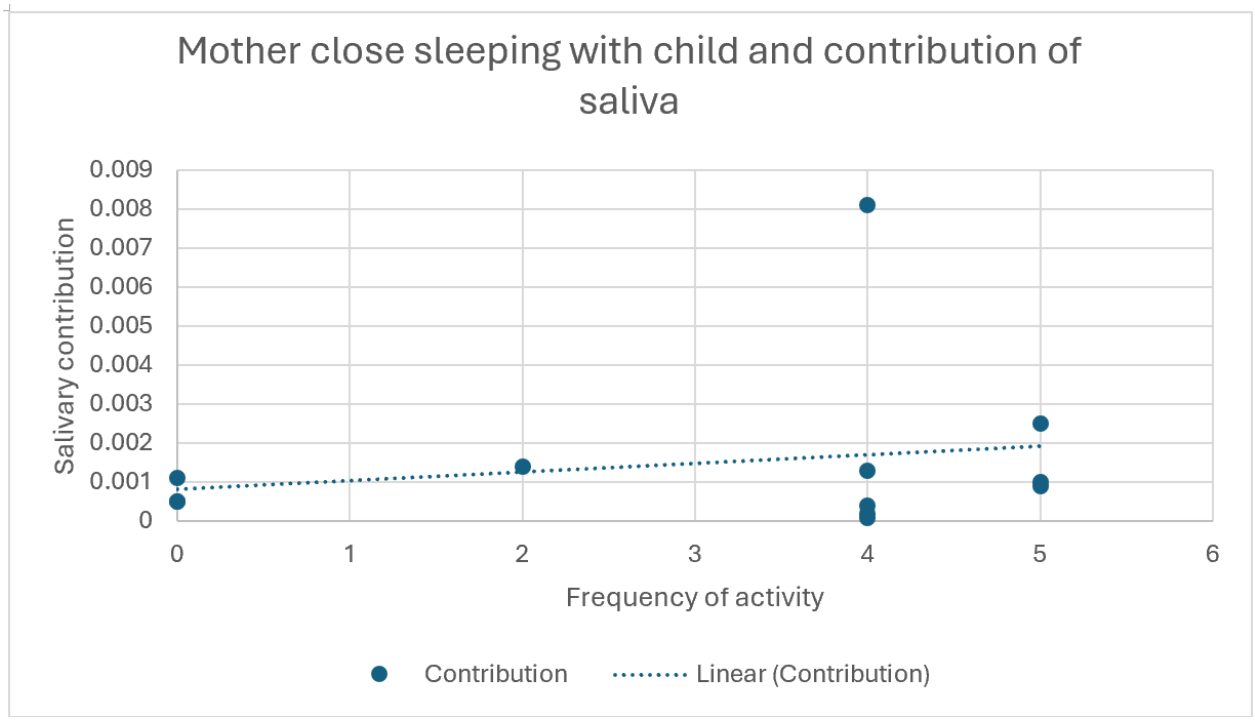


Figure 13: Scatterplot of the frequency of activity (mother close sleeping with child (x-axis) and the contribution of the saliva (y-axis, as a proportion).

This association was found to be statistically significant (p=0.0247, Spearman correlation test), Rho=0.641.

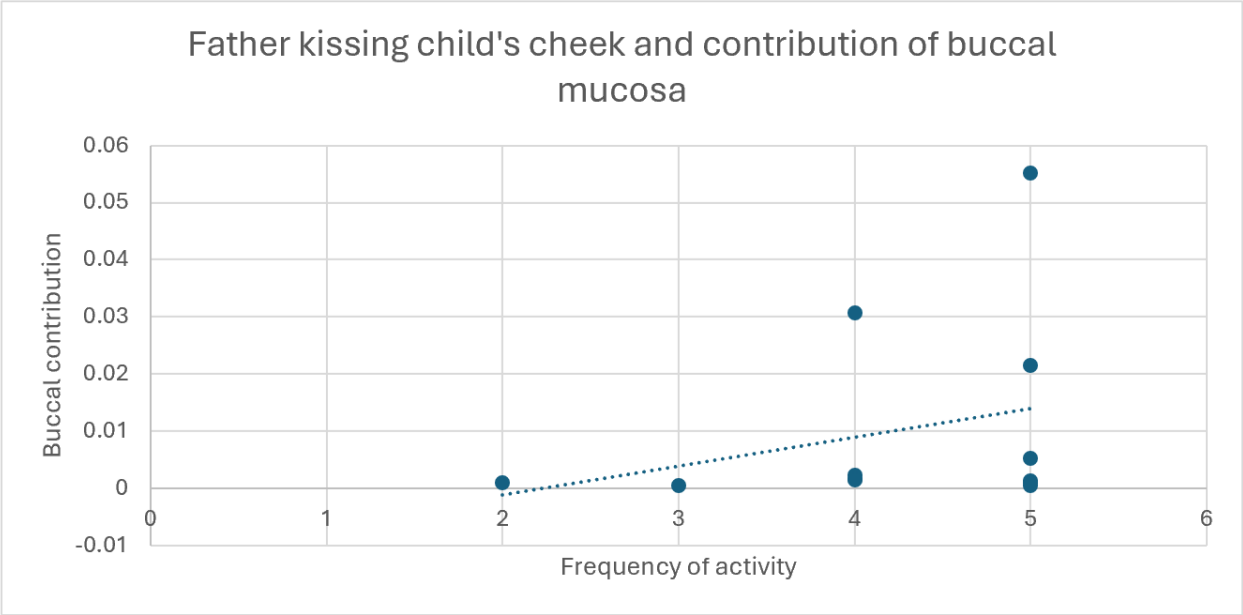


Figure 14: Scatterplot of the frequency of activity (father kissing the child's cheek (x-axis) and the contribution of the buccal mucosa (y-axis, proportion).

This association was found to be statistically significant (p=0.009, Spearman correlation test). Rho=0.714.

Chapter 5: DISCUSSION

In 1985, Dr. Geoffrey Rose articulated the concept of the prevention paradox in his seminal paper, 'Sick Individuals and Sick Populations.' (Rose, 2001). He demonstrated that interventions benefiting an individual might have a minimal impact on the wider population, whereas a preventive measure that brings significant benefits to a community may offer little benefit to each individual. For example, water fluoridation reduces the overall caries rate across a population but might have a negligible benefit for any single person. Conversely, treating carious lesions in one individual benefits that individual directly, yet does not affect the caries incidence in the rest of the population. This dichotomy was recently resonant during the COVID-19 pandemic, where vaccinations benefitted the community at large through herd immunity, but a vaccinated person themself may still contract COVID-19, albeit with lesser intensity (Ronchini et al., 2022). This illustrates that a community-based risk reduction often requires the participation of many to significantly reduce the risk for a few. It underscores the importance for physicians and epidemiologists to understand the “reference frame” by which interventions should be performed, which then enables them to effectively target problems from various angles. Current therapeutic approaches to treating periodontal diseases target the individual themselves, with the idea that there are minimal contributions from outside sources. Our research estimates that 8% of the recolonizing subgingival microbiota is from outside sources. Due to the close similarity between sub- and supra-gingival plaque, we believe that the contribution of easily colonizable microbiota should be considered as a median of 12%, which is the sum of the contribution of these two sources. This contribution from external sources, often from those living in close proximity to an individual, warrants consideration that perhaps treating periodontal disease is not an individual matter, but rather a family, or community matter. Periodontal disease is a complex, multi-factorial, microbially driven biofilm-based disease, and our research further validates previous research (Alanazi, 2022; Asikainen, 1999; Brito, 2019; Könönen, 2000; Monteiro, 2021; Reis, 2023; Tuite-McDonnell, 1997; Valles-Colomer, 2023; Van Winkelhoff, 2005; von Troil-Lindén, 1997; Von Troil-Lindén, 1995) in that oral microbiota is transmissible from one individual to another. Our study also quantified, for the first time, the fraction of the subgingival plaque attributable to transmissibility between family members.

This study agrees with previous research in that oral microbes are driven, for the most part, by the ecological niche of that location (Fine et al., 2013). In this study, the dissimilarity of all samples, as shown by the PCoA plots, showed that ecological niche was the main driver of sample dissimilarity. This is congruent with the findings of other research groups (Human Microbiome Project, 2012). In fact, we re-analyzed the publicly available data from that study (appendix figure A) to understand the uniqueness of our samples. Our study is in agreement with the results of HMP, in that differences are primarily driven by oral niches, and that the subgingival and supragingival plaque have a different microbial composition compared to the other oral niches. While HMP data showed this separation while clustering bacteria into operational taxonomic units (OTUs), our study demonstrates that this relationship is sustained even at an amplicon sequence variant (ASV) level. It also expands current knowledge by demonstrating that familial allocation and individual relationships are secondary drivers of separation. This may explain why genetically unrelated individuals can still colonize the microbiome of others. In a study examining the development and maturation of the oral microbiome, the authors (Mason et al., 2018) noted the same thing, namely, that the ecological niche was the main driver in determining species richness. With the eruption of teeth into the oral cavity, new habitats are created, with complex and specific niches. The greatest influx of species richness in the oral cavity occurs with the first tooth's eruption (Mason, 2018), introducing a novel and complex habitat for suitable bacteria.

Similar to the current study, von Troil-Lindén et al. (1996) investigated the re-establishment of the oral microbiome before and after periodontal treatment. In the von Troil-Lindén et al. (1996) study, the authors investigated 7 periodontitis patients, 6 spouses, and no children (von Troil-Lindén et al., 1996). These authors found that most periodontopathogens found 6 months after periodontal treatment were self-inoculated. Approximately 65% of the subgingival microbiome came from sources within the self (von Troil-Lindén et al., 1996). Our study refined this finding even further: 56% of the subgingival bacteria in the child can be attributed to specific sources (46% from subgingival sources, 9% from supragingival sources, and 1% from buccal mucosa). Furthermore, we showed that external sources also contributed to the re-establishment of the subgingival microbiome, including 8% from siblings and 3% each from mother and father, respectively. With even further resolution, we showed that 8% of the subgingival microbiome came from subgingival

sources of other family members, 4% came from supragingival sources of other family members, and 1% from the buccal mucosa of other family members.

Interestingly, the aforementioned Mars research group (Bacci et al., 2021) suggested that the salivary microbiome showed little transmission between, and little effect on, the microbiome of individuals living in close quarters. When the participants entered the isolated environment, the authors found that the salivary bacterial richness of each individual crew member decreased. Quantitative differences between crew members, however, remained relatively unaltered, suggesting that crew members did not meaningfully exchange salivary microbiota, despite having qualitative differences in the salivary microbial composition across different time points. The changes seen in each crew member were driven by a personal and unique set of microbes. Although this seems to contrast with the results of the current study, this contrast is not surprising. First of all, the Bacci study only investigated salivary samples and similarity. As shown in our current study, microbial differences are driven by ecological niches. Saliva is the conduit, or the vehicle of transmission, and even very small amounts of contributing bacteria in the saliva can be effectively transferred between individuals. This was shown in the fact that only 0.8% of the bacteria in saliva are from the subgingival plaque, and yet subgingival plaque bacteria account for 8% (the greatest external source) of the contributions to the subgingival bacteria of the child as the sink.

In this study, we investigated the effect of close contact activities on the transmission of oral microbes on the subgingival recolonization in the child. In a longitudinal twin study, Freire et al. (Marcelo Freire et al., 2020) suggested that the oral microbiome is shaped mostly by environment rather than genetics. In our study, we saw that close sleeping with the child and cuddling with the child by the mother, as well as kissing on the child's cheek by the father has a statistically significant contribution to the oral microbiome of the child. Könönen (Könönen, 2000) suggested that frequent contacts, adequate inoculation size, suitable ecological niche, and a receptive period (among other things) may be important in contributing to the transmission of oral microbes. In another study by Van Winkelhoff (Van Winkelhoff & Boutaga, 2005), the authors suggested that cohabitation with an infected individual increases this rate of transmission. Our study confirms these observations and postulates that the transmissibility of subgingival bacteria is relatively powerful despite being a small percentage of salivary microbiota.

In our study the contribution of the siblings (8%) appeared to have a greater effect than both mother and father (3% each, respectively) on the recolonization of the subgingival microbiome of the child. This differs slightly from what has been seen or suggested in another study. In one study, Mason et al. (Mason et al., 2018) showed that initially a child's oral microbiome is shared relatively closely with the mother (father and siblings were not investigated). As the child matures, it appears that the child develops a more unique and divergent oral microbiome, perhaps due to exposure to more individuals and increase in complexity of the shared environment. Our current study suggests that the contribution from siblings may be more important than previously expected, perhaps even more so than the contribution of mother as time moves on. We believe this may have the potential to contribute to a paradigm shift in the concept of horizontal transmission of the oral microbiome in families, shedding light on the importance of sibling-to-sibling transmission of oral microbes.

Previous studies (Asikainen & Chen, 1999; Asikainen et al., 1987; Brito et al., 2019; Van Winkelhoff & Boutaga, 2005) have shown the transmission of oral microbes in both mother-child dyads and within family units, including between spouses as well as between parents and children. As discussed previously, *A. actinomycetemcomitans* and *P. gingivalis* display different transmission routes between family members (Van Winkelhoff & Boutaga, 2005). Although our study did not look specifically at what microbes tend to show higher transmissibility or their routes of transmission between different individuals, we believe this question has been meaningfully examined using saliva in a study with large sample size (n=126 mother/child dyad) (Valles-Colomer, 2023). Gram-staining of the bacteria was significantly associated with household transmission within the household (p=0.04). Gram-negative bacteria, which are generally more resistant to breakdown by use of sanitizers, had higher transmissibility than Gram-positive bacteria. Interestingly, the same study found that aerotolerance was not significantly associated with enhanced transmissibility. Since most of the microbiota in the subgingival plaque are oxygen-sensitive in states of periodontal disease, this finding was surprising. Perhaps less surprising would be the expectation that these species are most likely transmitted *en-block* as supra-/sub-gingival biofilm fragments. This transmission provides them with a temporary respite from oxygen and free radical exposure, compared to if these microbiota were in their planktonic state, and therefore be in a more vulnerable state to the outside environment. Regardless of transmission route per species

or phenotypical properties of the microbiota, our study specifically shows that that both horizontal and vertical transmission of the oral microbiome are important contributors to the subgingival bacteria of a child.

This is the first study of its kind to investigate close contact activities in the family and the transmission of oral bacteria. Regarding the effect of close contact activities and lifestyle on the transmission of the oral microbiome, one study by Michael Freire (Freire, 2020) suggested that the age at which brushing started, as well as post-brushing activities, shaped microbial changes in twins. Few other studies have investigated the impact of close-contact activities on the transmission of the oral microbiome. In our study, we saw that three activities of those investigated were correlated with the transmission of the oral microbiome: mothers close sleeping with children (saliva), mothers cuddling with children (subgingival plaque), and fathers kissing the child on the cheek (buccal mucosa).

Several studies have shown the relationship between families and periodontal disease phenotype (Alanazi, Alturaif, et al., 2022; Mabelle Freitas Monteiro et al., 2021; Reis et al., 2023; Umeda et al., 2004; Van Winkelhoff & Boutaga, 2005). These authors suggested that children of parents with periodontal disease displayed a more pathogen rich biofilm compared to children of healthy parents. Furthermore, this dysbiotic-prone biofilm seemed to be relatively resilient, even after improvement in oral hygiene (Monteiro, 2021). Although our study did not investigate the disease status of family members, it provides evidence of the transmissibility of the oral microbiome, as we showed that the subgingival microflora is recolonized by internal and external sources to the child, including contributions from family members. Considering that 0.8% of the salivary microbiome was composed of bacteria from the subgingival plaque of the individual, and 8% of the external sources of the subgingival plaque of the child is the subgingival plaque of family members, this suggests high transmissibility of the subgingival bacteria through inter-personal transmission and subsequent recolonization of the subgingival niche. Therefore, we ascertain that the subgingival plaque is transmissible, and therefore any diseases caused by its microbial content must be communicable.

Chapter 6: CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusion

The global burden of diseases study has classified diseases as either communicable diseases or noncommunicable diseases (NCDs) (Mohan et al., 2019). The World Health Organization, the Joint Workshop by the European Federation of Periodontology (EFP) and the European arm of the World Organization of Family Doctors (WONCA Europe) consider the two most common oral diseases, dental caries and periodontitis, as noncommunicable diseases (Organization, 2020) (Herrera et al., 2023). Our study ascertains the growing body of evidence that suggests that subgingival plaque is transmissible, and as such using the term "non-communicable diseases" for periodontal diseases may mislead clinicians and patients.

The EFP clinical practice guidelines published after the World Workshop in 2017 puts patient care in the center of treatment without regard to familial interventions (Herrera et al., 2023; (Allen & Feigl, 2017). This patient-centered care, while necessary, is limited in perspective. The findings from our current study suggest that perhaps dental care providers should take a family perspective when treating an individual with periodontal disease.

According to our results, sources from within the person, and from other family members contribute to the recolonization of the subgingival microbiome. These external sources include microbes from mother, father, and most importantly, siblings. This is a shift in our understanding of familial transmission. The majority of previous studies (Asikainen, 1999; Brito, 2019; Monteiro, 2021; Nibali, 2019; Reis, 2023; Sulyanto, 2019; Valles-Colomer, 2023; Van Winkelhoff, 2005; von Troil-Lindén, 1996) focused on transmission from parent to child or between spouses. Our study suggests that each sibling contributes approximately 8% of the microbes to the recolonizing subgingival bacteria, compared to each parent (approximately 3%). As the number of siblings increases, so does the contribution. This study investigated further the microbial make-up of the subgingival microbiome and possible sources of transmission between individuals. Taken together, our study shows that each person in the household is a potential contributor to the overall subgingival microbiome, and that transmission should not be examined in a strict parent/child

transmission modality as was previously done. Moreover, it expands our knowledge on which key players affect the subgingival microbial recolonization.

Our study found that the oral niche is the main driver of microbial selection. This is in accordance with the principle of "everything is everywhere, but the environment selects" (de Wit & Bouvier, 2006). Saliva, being the conduit of transmission, carries shedding microbiota from different areas in the mouth, depending on the surface area. The tongue, with its multi-dimensional grooves and retention of microbiota was the highest contributor, followed by buccal mucosa, then supragingival plaque, then subgingival plaque. Nevertheless, supra and subgingival plaque contributed the highest inter-individual transmission. We attribute this finding to niche selection. This niche selection shows that the supra- and sub-gingival plaque microbiota are highly selective to microbiota that reside in these two niches, to the point that despite being a minor contributor to salivary microbiota, they are highly efficient at occupying the subgingival plaque of others.

Based on these results, we can conclude that oral bacteria are transmissible, and this transmission occurs within families: between spouses, from parent to child, and, most importantly, between siblings. Furthermore, apart from sources within the self, the subgingival bacteria of family members, especially siblings, are the greatest contributors to the subgingival recolonization of the youngest child.

6.2 Strengths and Contributions

The current study is one of very few studies that examined the effect of close contact activities on the transmission of the oral microbiome, and to our knowledge, the first study that investigated bacterial transmission using ASVs between all family members through source tracking. This increased resolution has provided us, for the first time, definitive, quantitative measurement of transmissibility of the subgingival plaque, and the sources that contribute to it.

The current study also shows that the contribution of siblings to the recolonization of the oral microbiome may be the most important factor, which is contrary to previous thinking in that mother and father were the most important contributors. The importance of the family unit in

recolonization of the microbiome of the child indicates that perhaps periodontal care should not only be patient-centered, but family-centered. This is the first study that demonstrates this point, and constitutes a paradigm shift in our understanding of oral microbial transmission.

6.3 Future directions

Future opportunities for investigation and increased understanding on this topic include:

Sample size: Similar studies should be performed and with greater sample size to corroborate the findings that we found herein to determine the repeatability and reliability of our results.

Disease status of participants should be assessed: It would be informative to investigate the re-establishment and transmission of the oral microbiome in the context of disease status of the individual. In future studies, examining the periodontal status of participants with a full examination, or through screening with the Community Periodontal Index of Treatment Needs (CPITN) on Ramfjord teeth, could give more insight as to any correlation between periodontal status and transmission of oral bacteria.

Better investigation of sibling activity: Future studies should investigate the relationship between close contact activities of siblings and the effect on transmission of the oral microbiome. Living arrangements, sleeping arrangements, and close contact activities could all be areas of investigation.

Longer follow-up period: Our participants were followed for one week. Longer follow-up time would provide further insight into the transmission, re-establishment, and maturation of the oral microbiome over time and in the context of family units.

Further investigation into family activities and demographics: Presently, very little literature exists regarding close contact activities within family units relating to possible transmission of the oral microbiome. Significant opportunities for increased understanding of different activities and their effect on transmission in different cultures and different family structures (one child,

single parent, multi-generational, etc.) exist. Furthermore, our study offers some preliminary findings that could be further investigated with larger and more diverse samples.

6.4 Limitations and Weaknesses

Some of the limitations and weaknesses of this study include:

Limited Sample Size: Fourteen families were recruited for this family, for a total of 71 participants (14 mothers, 14 fathers, and 43 children). This was a pilot study involving a small number of families, which may limit the generalizability of our findings. Future studies with larger sample sizes, different demographics, and family characteristics would improve the generalizability of the results.

Detailed questionnaire of the siblings: The finding that siblings demonstrated the highest level of subgingival transmission was a surprising finding in this study. It is also the first time such a finding has been demonstrated in periodontal literature. When we conceived the study, we expected the largest contribution to be from the parents, and especially the mother. The questionnaire, therefore, was directed at investigating the close contact activities of parents with the youngest child. We did not investigate the close contact activities of siblings with the youngest child. Given our findings about transmission between siblings, it will be important for future research to investigate further into this relationship.

Short Follow-Up Period: Our follow-up with the children lasted only one week. It is possible that changes in the microbiome might become more/less apparent over a longer period of follow up as the subgingival plaque matures.

Subgingival Scaling Not Performed: In the perturbation of the supra- and sub-gingival plaque, oral prophylaxis was performed on the youngest child. We did not perform scaling of any teeth, including subgingival scaling of the teeth. Since children had periodontal sulci ≤4 mm, we believe prophylaxis was effective at meaningfully disrupting the subgingival plaque. That being said, a thorough and complete debridement of all teeth for the youngest child may have been more

effective at disturbing the subgingival microbial environment to allow for a greater “reset” of said environment. A study that attempts that, while balancing the benefits/risks of such intervention, is warranted.

REFERENCES

Alanazi, L. M., jumah Alturaif, D., Alhassan, M. H., Alshahrani, A. M. S., Al-Ghamdi, R. J. S., Shabi, M. M. A., & Almalkie, R. A. (2022). Effect of Parental History of Periodontal Disease on Children. *Saudi J Oral Dent Res*, *7*(8), 192-200.

Allen, L. N., & Feigl, A. B. (2017). What's in a name? A call to reframe non-communicable diseases. *Lancet Glob Health*, *5*(2), e129-e130. https://doi.org/10.1016/S2214-109X(17)30001-3

Asikainen, S., & Chen, C. (1999). Oral ecology and person-to-person transmission of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. *Periodontol 2000*, *20*, 65-81. https://doi.org/10.1111/j.1600-0757.1999.tb00158.x

Asikainen, S., Jousimies-Somer, H., Kanervo, A., & Summanen, P. (1987). Certain bacterial species and morphotypes in localized juvenile periodontitis and in matched controls. *J Periodontol*, *58*(4), 224-230. https://doi.org/10.1902/jop.1987.58.4.224

Awany, D., & Chimusa, E. R. (2020). Heritability jointly explained by host genotype and microbiome: will improve traits prediction? *Briefings in Bioinformatics*, *22*(3). https://doi.org/10.1093/bib/bbaa175

Bacci, G., Mengoni, A., Emiliani, G., Chiellini, C., Cipriani, E. G., Bianconi, G., Canganella, F., & Fani, R. (2021). Defining the resilience of the human salivary microbiota by a 520-day longitudinal study in a confined environment: the Mars500 mission. *Microbiome*, *9*(1), 152. https://doi.org/10.1186/s40168-021-01070-5

Berger, D., Rakhamimova, A., Pollack, A., & Loewy, Z. (2018). Oral Biofilms: Development, Control, and Analysis. *High Throughput*, *7*(3). https://doi.org/10.3390/ht7030024

Brito, I. L., Gurry, T., Zhao, S., Huang, K., Young, S. K., Shea, T. P., Naisilisili, W., Jenkins, A. P., Jupiter, S. D., Gevers, D., & Alm, E. J. (2019). Transmission of human-associated microbiota along family and social networks. *Nature Microbiology*, *4*(6), 964-971. https://doi.org/10.1038/s41564-019-0409-6

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. https://doi.org/10.1038/nmeth.3869

de Wit, R., & Bouvier, T. (2006). 'Everything is everywhere, but, the environment selects'; what did Baas Becking and Beijerinck really say? *Environmental Microbiology*, *8*(4), 755-758. https://doi.org/10.1111/j.1462-2920.2006.01017.x

Demmitt, B. A., Corley, R. P., Huibregtse, B. M., Keller, M. C., Hewitt, J. K., McQueen, M. B., Knight, R., McDermott, I., & Krauter, K. S. (2017). Genetic influences on the human oral microbiome. *BMC Genomics*, *18*(1), 659. https://doi.org/10.1186/s12864-017-4008-8

Fine, D. H., Markowitz, K., Fairlie, K., Tischio-Bereski, D., Ferrendiz, J., Furgang, D., Paster, B. J., & Dewhirst, F. E. (2013). A consortium of Aggregatibacter actinomycetemcomitans, Streptococcus parasanguinis, and Filifactor alocis is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. *J Clin Microbiol*, *51*(9), 2850-2861. https://doi.org/10.1128/jcm.00729-13

Freire, M., Moustafa, A., Harkins, D. M., Torralba, M. G., Zhang, Y., Leong, P., Saffery, R., Bockmann, M., Kuelbs, C., Hughes, T., Craig, J. M., & Nelson, K. E. (2020). Longitudinal Study of Oral Microbiome Variation in Twins. *Sci Rep*, *10*(1), 7954. https://doi.org/10.1038/s41598-020-64747-1

Freire, M., Moustafa, A., Harkins, D. M., Torralba, M. G., Zhang, Y., Leong, P., Saffery, R., Bockmann, M., Kuelbs, C., Hughes, T., Craig, J. M., & Nelson, K. E. (2020). Longitudinal Study of Oral Microbiome Variation in Twins. *Scientific Reports*, *10*(1), 7954. https://doi.org/10.1038/s41598-020-64747-1

Herrera, D., Sanz, M., Shapira, L., Brotons, C., Chapple, I., Frese, T., Graziani, F., Hobbs, F. D. R., Huck, O., Hummers, E., Jepsen, S., Kravtchenko, O., Madianos, P., Molina, A., Ungan, M., Vilaseca, J., Windak, A., & Vinker, S. (2023). Association between periodontal diseases and cardiovascular diseases, diabetes and respiratory diseases: Consensus report of the Joint Workshop by the European Federation of Periodontology (EFP) and the European arm of the World Organization of Family Doctors (WONCA Europe). *J Clin Periodontol*, *50*(6), 819-841. https://doi.org/10.1111/jcpe.13807

Human Microbiome Project, C. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207-214. https://doi.org/10.1038/nature11234

Knights, D., Kuczynski, J., Charlson, E. S., Zaneveld, J., Mozer, M. C., Collman, R. G., Bushman, F. D., Knight, R., & Kelley, S. T. (2011). Bayesian community-wide culture-independent microbial source tracking. *Nature Methods*, *8*(9), 761-763. https://doi.org/10.1038/nmeth.1650

Könönen, E. (2000). Development of oral bacterial flora in young children. *Annals of Medicine*, *32*(2), 107-112. https://doi.org/10.3109/07853890009011759

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, *17*(1), 10-12. https://doi.org/10.14806/ej.17.1.200

Mason, M. R., Chambers, S., Dabdoub, S. M., Thikkurissy, S., & Kumar, P. S. (2018). Characterizing oral microbial communities across dentition states and colonization niches. *Microbiome*, *6*(1), 67. https://doi.org/10.1186/s40168-018-0443-2

Mohan, P., Mohan, S. B., & Dutta, M. (2019). Communicable or noncommunicable diseases? Building strong primary health care systems to address double burden of disease in India. *J Family Med Prim Care*, *8*(2), 326-329. https://doi.org/10.4103/jfmpc.jfmpc_67_19

Monteiro, M. F., Altabtbaei, K., Kumar, P. S., Casati, M. Z., Ruiz, K. G. S., Sallum, E. A., Nociti-Junior, F. H., & Casarin, R. C. V. (2021). Parents with periodontitis impact the subgingival colonization of their offspring. *Sci Rep*, *11*(1), 1357. https://doi.org/10.1038/s41598-020-80372-4

Monteiro, M. F., Casati, M. Z., Sallum, E. A., Silverio, K. G., Nociti-Jr, F. H., & Casarin, R. C. V. (2022). The familial trend of the local inflammatory response in periodontal disease. *Oral Diseases*, *28*(1), 202-209. https://doi.org/10.1111/odi.13738

Monteiro, M. F., Casati, M. Z., Taiete, T., Sallum, E. A., Nociti, F. H., Jr., Ruiz, K. G., & Casarin, R. C. (2014). Salivary carriage of periodontal pathogens in generalized aggressive periodontitis families. *International Journal of Paediatric Dentistry*, *24*(2), 113-121. https://doi.org/10.1111/ipd.12035

Nibali, L., Bayliss-Chapman, J., Almofareh, S. A., Zhou, Y., Divaris, K., & Vieira, A. R. (2019). What Is the Heritability of Periodontitis? A Systematic Review. *J Dent Res*, *98*(6), 632-641. https://doi.org/10.1177/0022034519842510

Organization, W. H. (2020). Achieving better oral health as part of the universal health coverage and noncommunicable disease agendas towards 2030. *Report to Director General*.

Page, R. C., & Kornman, K. S. (1997). The pathogenesis of human periodontitis: an introduction. *Periodontol 2000*, *14*, 9-11. https://doi.org/10.1111/j.1600-0757.1997.tb00189.x

Piovani, D., Nikolopoulos, G. K., & Bonovas, S. (2022). Non-Communicable Diseases: The Invisible Epidemic. *J Clin Med*, *11*(19). https://doi.org/10.3390/jcm11195939

Reis, A. A., Monteiro, M. F., Bonilha, G. M., Saraiva, L., Araújo, C., Santamaria, M. P., Casati, M. Z., Kumar, P., & Casarin, R. C. V. (2023). Parents with periodontitis drive the early acquisition of dysbiotic microbiomes in their offspring. *J Clin Periodontol*, *50*(7), 890-904. https://doi.org/10.1111/jcpe.13815

Shen, W., Le, S., Li, Y., & Hu, F. (2016). SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q File Manipulation. *PLoS One*, *11*(10), e0163962. https://doi.org/10.1371/journal.pone.0163962

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

Umeda, M., Miwa, Z., Takeuchi, Y., Ishizuka, M., Huang, Y., Noguchi, K., Tanaka, M., Takagi, Y., & Ishikawa, I. (2004). The distribution of periodontopathic bacteria among Japanese children and their parents. *J Periodontal Res*, *39*(6), 398-404. https://doi.org/10.1111/j.1600-0765.2004.00754.x

Valles-Colomer, M., Blanco-Míguez, A., Manghi, P., Asnicar, F., Dubois, L., Golzato, D., Armanini, F., Cumbo, F., Huang, K. D., Manara, S., Masetti, G., Pinto, F., Piperni, E., Punčochář, M., Ricci, L., Zolfo, M., Farrant, O., Goncalves, A., Selma-Royo, M., . . . Segata, N. (2023). The person-to-person transmission landscape of the gut and oral microbiomes. *Nature*, *614*(7946), 125-135. https://doi.org/10.1038/s41586-022-05620-1

Van Winkelhoff, A. J., & Boutaga, K. (2005). Transmission of periodontal bacteria and models of infection. *J Clin Periodontol*, *32 Suppl 6*, 16-27. https://doi.org/10.1111/j.1600-051X.2005.00805.x

von Troil-Lindén, B., Alaluusua, S., Wolf, J., Jousimies-Somer, H., Torppa, J., & Asikainen, S. (1997). Periodontitis patient and the spouse: periodontal bacteria before and after treatment. *Journal of Clinical Periodontology*, *24*(12), 893-899. https://doi.org/https://doi.org/10.1111/j.1600-051X.1997.tb01208.x

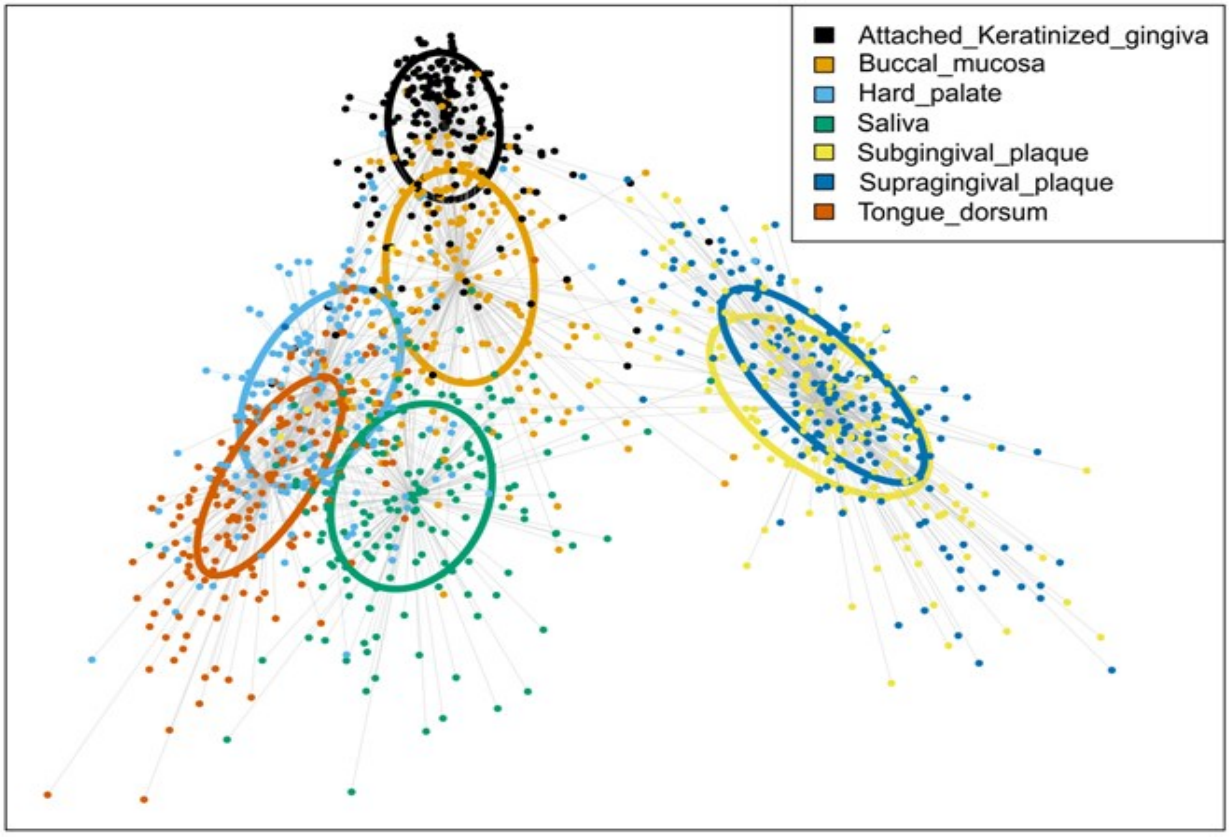
von Troil-Lindén, B., Saarela, M., Mättö, J., Alaluusua, S., Jousimies-Somer, H., & Asikainen, S. (1996). Source of suspected periodontal pathogens re-emerging after periodontal treatment. *J Clin Periodontol*, *23*(6), 601-607. https://doi.org/10.1111/j.1600-051x.1996.tb01831.x

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. https://doi.org/10.1101/610394

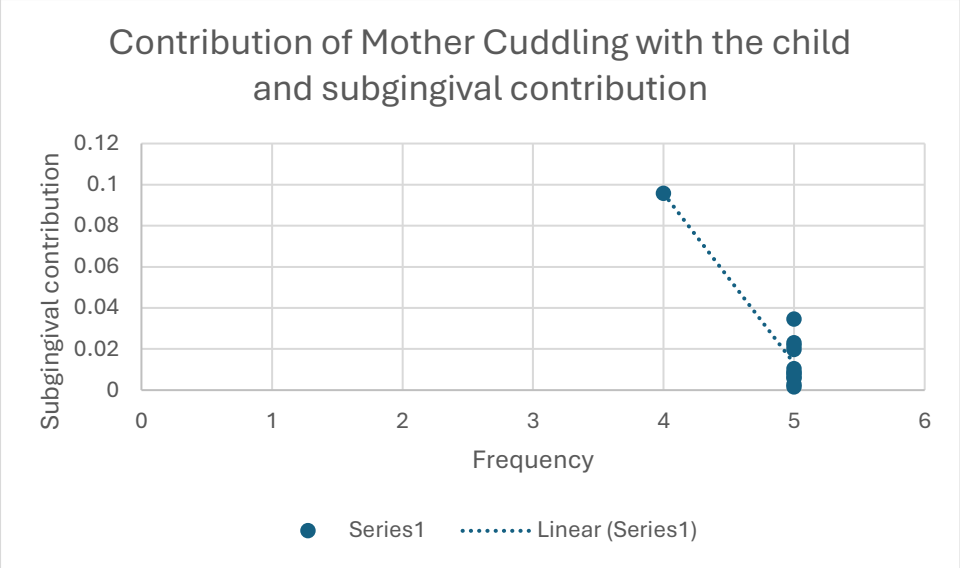
Wolf, T. G., Cagetti, M. G., Fisher, J. M., Seeberger, G. K., & Campus, G. (2021). Non-communicable Diseases and Oral Health: An Overview. *Front Oral Health*, *2*, 725460. https://doi.org/10.3389/froh.2021.725460

Appendix

Appendix I



Supplementary figure A: PCoA of PhILR dissimilarity of the Human Microbiome Project data (Human Microbiome Project, 2012). Publicly available data is of unrelated healthy individuals. Data is represented as OTUs, which cluster DNA sequences based on similarity. Circles represent one standard deviation of spread from the centroid.



Supplementary figure B: Scatterplot of the frequency of activity (mother cuddling with child (x-axis) and the contribution of the saliva (y-axis) as a proportion). This association was found to be statistically significant (p=0.010, Spearman correlation test), Rho=0.707, however, all responses were the same except one.